The effects of microplastic ingestion and environmental warming on camouflage and growth in common shore crabs

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Abstract

Anthropogenic stressors are becoming increasingly prevalent in the marine environment, both as direct pollutants (e.g. microplastics and noise), and indirectly through climate change (e.g. environmental warming and ocean acidification). Microplastics in particular are considered to be hazardous, due to their bioavailability through primary (diet), and secondary (respiration) means. This is owed to their small size (<5mm), ubiquity in the marine environment, and close resemblance of small prey items. Plastic pollution also frequently co-occurs with other ecological stressors, such as environmental warming through climate change. There is a growing body of evidence to suggest that exposure to multiple interacting stressors can magnify their adverse effects. However, little research exists on how stressors such as environmental warming and microplastics affect juvenile marine invertebrates, what this means for subsequent life-stages, and what coping mechanisms they may possess. Furthermore, stressor effects on antipredator behaviours such as colour change for camouflage have also received very little attention, despite these behaviours being pervasive among aquatic species. This limits our ability to make predictions on the impacts of stressor exposure on marine species, and what the subsequent implications are for their survival. Here, I address these knowledge gaps through a series of laboratory-based feeding studies, using environmentally relevant quantities of microplastics (0.5% by feed weight), and two temperature treatments 14°C (ambient environmental temperature) and 24°C (unseasonably high environmental temperature) on juvenile shore crabs (*Carcinus maenas*).

In Chapter 2, I examine the effects of microplastic ingestion as a singular stressor on camouflage efficacy and growth in juvenile shore crabs. Individuals were exposed to microplastic particles through feed over a period of 8 weeks. Weight, incidence of moulting, and carapace diameter were recorded on a weekly basis as proxies for juvenile growth. The level of luminance change (brightness) and subsequent camouflage were quantified in an ecologically relevant context using digital photo analysis, and a model of avian predator vision. Microplastic ingestion alone did not affect luminance change, and subsequent background matching in juvenile shore crabs. Additionally, it was also not found to significantly affect the incidence of moulting, or growth (weight change and carapace diameter). It however found that

juvenile shore crabs possess the capacity to remove sequestered microplastics from their gill surface through the process of moulting, thereby mitigating the possible negative effects of microplastic ingestion.

In Chapter 3 I build upon the findings of Chapter 2, and investigate the effects of combined stressors (microplastic ingestion and environmental warming) on camouflage and growth using the same methodology. The level of growth per moult (carapace diameter and weight) was found to be significantly reduced in individuals exposed to combined stressors. There was also an initial delay in moulting, as well as an overall reduction in moult frequency. However, the level of luminance change and camouflage remained unaffected by exposure to combined stressors, with any changes being attributed to exposure to a warmer thermal environment. Overall, this thesis indicates that common, co-occurring marine stressors have the capacity to interfere with fundamental physiological processes in marine species when stressors interact. Furthermore it suggests there are potential implications for future fitness and survival in juveniles.

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And finally, to those that unfortunately were not able to see me finish this research – Grandad, I'm sorry you didn't get to see the final copy, I hope this makes you proud. Sarah, I finally got rid of the crabs that you so mercilessly ripped me for.

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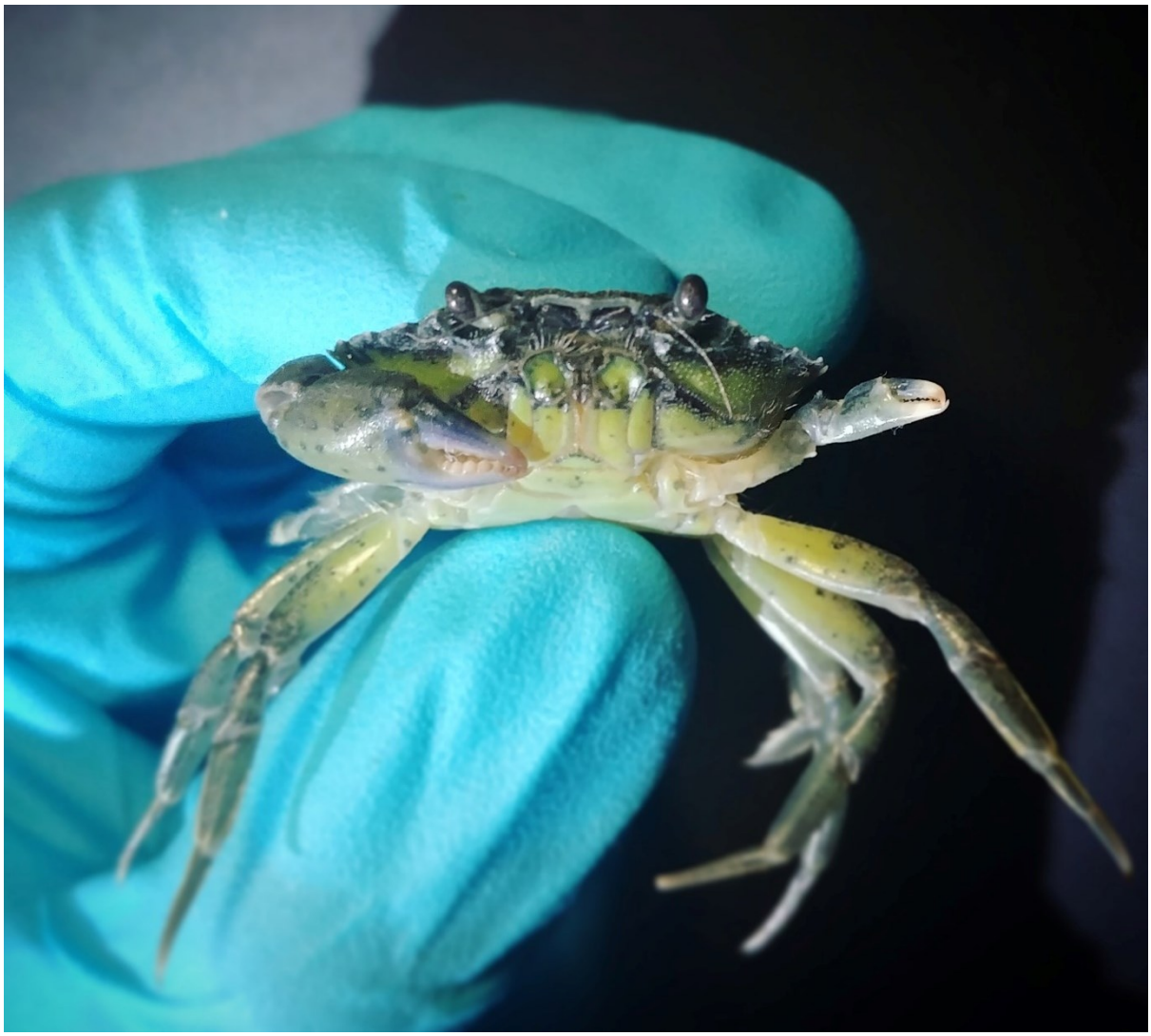


The Impact of Anthropogenic activity on Camouflage Efficacy

Camouflage is a widespread antipredator strategy, and is often considered the first line of defence for many species. It enables an organism to be overlooked or misidentified by resembling an aspect of the immediate visual environment (Hughes *et al.,* 2019). Environments typically undergo some form of temporal or spatial variation, with most species likely to encounter multiple habitats over their lifetime (Nokelainen *et al.,* 2017). As such species tend to exhibit some degree of flexibility in their camouflage to ensure maximal camouflage efficacy, which influences fitness by increasing survival probability (Zimova *et al.,* 2016). However, environmental variation can be altered by anthropogenic activity, from decreasing the time over which these changes occur, to altering the composition and interactions of abiotic factors (Mitchell

et al., 2006; Doney *et al.,* 2012; Lewis & Santos, 2016; Wu *et al.,* 2017; Richmond *et al.,* 2018). This thesis primarily focuses on microplastic pollution and climate change derived environmental warming as anthropogenic stressors. Although these issues have recently received increasing attention as separate concerns (Laist, 1997; Pörtner, 2010; Schulte *et al.,* 2011; Baulch & Perry, 2014; Lavers *et al.,* 2014; Watts *et al.,* 2014), few studies examine their effects on invertebrate species in the context of antipredator behaviours such as camouflage.. Fewer still, address these in the context of multiple stressors. This thesis addresses these knowledge gaps through a series of laboratory-based feeding studies, using environmentally relevant quantities of microplastics (approximately 734 particles $x10^3$ m^{-3}), and environmental warming simulated as two temperature treatments 14°C (ambient environmental temperature), and 24°C (unseasonably high environmental temperature) on juvenile shore crabs (*Carcinus maenas*).

Camouflage and Plasticity

The purpose of visual camouflage is to minimise the appearance of feature variation between an animal and its chosen surroundings, in most cases to avoid detection or recognition by visually guided predators (Merilaita & Stevens, 2011). Many forms of camouflage exist in the natural world, including masquerade (reduces recognition), background matching, and disruptive colouration (both reduce detection) (Stevens & Merilaita, 2009). Successful camouflage generally involves the process of phenotype matching to the individual's given environment. Typically, the phenotypes in question are hue, brightness, colour, pattern, and texture (Thayer, 1986; Nokelainen *et al*., 2017; Michalis *et al.,* 2017). However, most species exhibit some degree of plasticity in their phenotype to prevent a decrease in camouflage efficacy, which influences fitness through reducing their survival probability (Zimova *et al.,* 2016).

Phenotypic plasticity entails an individual altering a phenotype (e.g. behaviour, morphology, physiology, growth, life history) to suit the present environmental conditions in order to maximise their fitness (West-Eberhard, 1989; Young *et al*., 2003). The degree to which the exhibited plasticity is considered adaptive or non-adaptive, depends on the proximity of the phenotype to the local optimum (Ghalambor *et al.,* 2015). Environmental changes that can induce plasticity include biotic factors such as changes in herbivory, predation, and competition, or abiotic changes in

temperature and light (Miner *et al.,* 2005). These factors are also liable to anthropogenic influence, which can alter the speed and intensity at which these changes occur. Plasticity has an important role to play in adaptation to human-induced environmental changes, such as climate change. However, different types of plasticity act on various timescales, and not all plasticity is adaptive (Fox *et al.,* 2019).

The speed and degree of plasticity achievable is primarily dependent on the species utilising them (e.g. their physiological capacity), and the variability of their environments both spatially and temporally (Cuthill & Troscianko, 2011). Therefore, a fixed phenotype is unlikely to provide optimal camouflage over the lifespan of an individual, so individuals may instead adjust their appearance through reversible phenotypic plasticity, or ontogenetic changes (Duarte *et al.,* 2017; Stevens & Ruxton, 2019). The constraints on phenotypic changes usually derive from the makeup of the epidermis, which frequently may either lack in specialised pigment cells to produce phenotypic variation, or nervous control to enact rapid colour change. Other constraints include the composition of the integument (e.g. hair or feather), whose pigment could be fixed (e.g. cuttlefish)at the time of growth and undergo seasonal moulting to generate changes in phenotype (e.g. colour) (Cuthill & Trosianko, 2011).

In arthropod species, the process of moulting occurs frequently (particularly in growing juveniles), allowing dramatic changes in colouration to occur over a period of weeks (Stevens *et al.,* 2014). Despite the potential limitations of an exoskeleton, arthropods have the capacity to undergo both physiological and morphological changes (Umbers *et al.,* 2014). Even if these features do not limit plasticity, the process of undergoing phenotypic change itself is regarded as energetically costly (Talloen, *et al.,* 2004; Bergstrom *et al.,* 2012; Rogers *et al.,* 2013). This process involves balancing the growth of new tissues, and the up/down regulation of pigments against basal metabolic functions, with respect to their energy budgets (Duarte *et al*., 2017). As energetic budgets are typically limited, this is achieved through increasing the intake of energy (e.g. food), and minimising energy losses (e.g. reducing movement). Naturally, this assumes an abundant food supply and minimal risk of conspecific or predator interaction. However, this is increasingly not the case due to anthropogenic activity reducing suitable habitat availability and size (Tittensor *et al.,* 2010).

Colour Change

Colour change mechanisms can be broadly placed into two categories: morphological and physiological. Physiological changes occur over a period of milliseconds to hours, whereas morphological changes are far slower and take place over days to weeks (Umbers *et al.,* 2014). These changes occur via separate complex mechanisms such as pigment anabolism and catabolism (morphological), and chromatophore pigment migration (physiological) (Siegenthaler *et al.,* 2018). As such, colour change is frequently utilised to enhance an individual's camouflage and reduce their risk of detection by visually hunting predators.

Background matching (also referred to as crypsis), is among the most common of antipredator strategies to employ colour change as the primary mechanism to optimise camouflage (Stevens & Merilaita, 2009). The process of background matching involves individuals actively possessing colouration or patterning that closely resembles that of their microhabitat (Kang *et al.,* 2015). The most notable example of background matching through physiological means can be found in cephalopod species, which are particularly adept at making rapid changes in appearance that incorporate a mixture of colour, hue, luminance, and texture within seconds (Hanlon *et al*., 2008). The speed and complexity of these changes provides the individualwith camouflage efficient enough to conceal its identity from predatory di- and trichromatic fish species (Chiao *et al.,* 2011). However, the ability to adopt such large changes in phenotype rapidly is considered uncommon within the natural world, with phenotypic changes typically occurring over longer periods (Stevens *et al*., 2014). Lengthy morphological colour changes are common among crustaceans such as the American lobster (*Homarus amercanus*), which changes colour in response to environmental cues (e.g. background colouration and UV light) over a period of weeks (Tlusty *et al*., 2010).

Many species of crustaceans exhibit highly variable phenotypes in terms of carapace colouration, pattern, and luminance throughout their juvenile life-stages (Wade *et al*., 2008; Umbers *et al*., 2014; Stevens, 2016). Some of the most notable phenotypic variation has been found amongst juvenile shore crabs (*Carcinus maenas*) (Stevens *et al.,* 2014). Nokelainen *et al.,* (2019) found that juvenile variation in phenotypes reflect the complexity of the environments from which they derive. For example,

juveniles that originate from mudflat environments are typically more uniform in colour and adopt background matching as their primary antipredator defence (Stevens, 2016). In highly variable rockpool environments however, individuals exhibit more contrasting and variable patterning and colouration, which is more in line with disruptive colouration to impair predator search image formation (Bond, 2007; Nokelainen *et al.,* 2019; Price *et al.,* 2019). However, the intraspecific diversity of juvenile shore crab appearance declines with age and size, as adults adopt a more generalist camouflage and assume their iconic green colouration (Nokelainen *et al.,* 2019). This ontogenetic change relates to adult shore crabs moving away from shallow intertidal nursery habitats, towards deeper waters such as seagrass beds (McGaw *et al.,* 1992). Moult frequency also decreases with age, resulting in a shift in antipredator defences towards increased in defensibility through a thickening of the carapace and aggressive behaviour (Crothers, 1967; Souza *et al.,* 2011).

Stressors in the Marine Environment

Marine ecosystems are increasingly under pressure from anthropogenic activity, particularly those in coastal areas with high levels of human development (Doney *et al.,* 2012; Wu *et al.,* 2017). Human activity brings with it a variety of ecological stressors such as sedimentation, eutrophication, chemical pollution, noise pollution, plastic pollution, and overfishing (Lewis & Santos, 2016; Richmond *et al.,* 2018). These can generate a range of negative effects from the level of the individual (e.g. reduced body condition and energy reserves, decreased camouflage efficacy, and delayed escape responses), to population level effects (e.g. declines in juvenile recruitment into adulthood, and population fragmentation) (Wright *et al.,* 2013; Cole *et al.*, 2015; Watts *et al.,* 2016; Carter *et al.,* 2020). In areas of high anthropogenic activity cross-over between ecological stressors is not uncommon, with interactions ranging from additive, to synergistic, and antagonistic. Human activity also has the capacity to affect the marine environment indirectly by driving climate change induced environmental perturbation (Mitchell *et al.,* 2006).

While abiotic conditions (e.g., temperature and pH) regularly fluctuate in coastal environments, climate change is increasing the frequency and intensity of these fluctuations (Mitchell *et al.,* 2006; IPCC, 2013; IPCC, 2014). This increases the likelihood of exceeding the intensity thresholds of even the hardiest species, and inducing physiological stress (Bernhardt & Leslie, 2013). Acute and chronic physiological stress can result in significant shortfalls in cardiac performance, leading to an inadequate provisioning of oxygen and subsequently affecting energy allocation and reserves (Somero, 2010). These changes in abiotic conditions are also associated with population level shifts such as altered dispersal patterns, changes in species interactions (e.g. predator-prey), and community composition (Doney *et al.,* 2012). This can have knock-on effects for local biodiversity which plays a key role in maintaining a range of ecosystem services, which not only benefit the marine ecosystems within which they occur, but also to humans who derive economic utility from this diversity (Hanley, 2016).

Plastic as a Global Pollutant

Plastic pollution is a rapidly growing environmental and economic concern on a global scale (Horton & Barnes, 2020). The most commonly sampled polymers are polyethylene, polypropylene, and polyvinylchloride in freshwater and marine environments (Li *et al.,* 2017; Conkle *et al*., 2018; Lindeque *et al*., 2020). Plastic particles are readily classified into three size categories: macro (>5mm), micro (<5mm), and nano (<1µm). It is estimated that up to 12.7 million tons of plastic enters the marine environment on an annual basis (Jambeck *et al.,* 2015). However, there is increasing evidence to suggest that environmental concentrations may in fact be underestimated (Conkle *et al*., 2018; Lindeque *et al*., 2020). This is particularly true of smaller microplastics and nanoplastics, which may be omitted from surveys due to being smaller than the conventional mesh size used in trawls (333 µm) (Li *et al.,* 2017). This is supported by Norén (2007), who found that there are around 100,000 times more plastic particles collected when using an 80µm mesh net, than a 450µm in the coastal waters of Sweden. However, it is important to note that there are large spatiotemporal variations in plastic particle distribution which may inhibit accurate estimation (Law *et al.,*2014).

Microplastics and nanoplastics originate from a variety of sources, most notably through the fragmentation of larger items as a result of environmental exposure (e.g. physical abrasion, UV degradation, photo-oxidation, biological activity, and wave action) (Andrady, 2003; Thompson *et al.,* 2004; Barnes *et al.,* 2009; Hidalgo-Ruz *et al.,* 2012; Courtene-Jones *et al*., 2018). Microplastics are considered to be the most predominant form of plastic pollution in the marine environment (Thompson *et al.,* 2004; Conkle *et al.,* 2018), accounting for around 92% of debris found at the ocean surface (Eriksen *et al.,* 2014). In addition to secondary fragments, microplastics also commonly take the form of primary plastics such as nurdles (pre-production industrial pellets), cosmetic microbeads, and synthetic textile fibres (Conkle *et al.,* 2018). The ubiquity of these particles in the aquatic environment (e.g. sediments, the water column and surface), means that particle exposure is likely to be a constant feature through all life stages of aquatic species (Thompson *et al.,* 2004; Barnes *et al.,* 2009; Li *et al.,* 2017).

The most notable interactions between marine biota and plastics are through entanglement and ingestion (Laist, 1997; Baulch & Perry, 2014; Lavers *et al.,* 2014; Duncan *et al.,* 2018). Micro and nanoplastics frequently resemble common prey items through their overlapping size, shape, and colouration, making them increasingly bioavailable to a variety of organisms (Wright *et al.,* 2013; Galloway *et al.,* 2017; Bradney *et al*., 2019). For example, Botterell *et al*., (2020) found that microbeads were preferentially selected over other shapes (e.g. fragments and fibres) by European lobster (*Homarus Gammarus*) larvae. This indicates that this shape may be particularly bioavailable to larger predatory invertebrate species, over species that are smaller or utilise alternative feeding strategies such as *Calanus helgolandicus* which preferentially ingested microfibres (Botterell *et al.,* 2020). A consequence of plastic particle ingestion is false satiation through blockage of the digestive tract. This holds implications not only for the general health and body condition of the individual, but may also pose subsequent energetic restrictions for future life-history traits (e.g. growth and fecundity) (Ferreira de Barros *et al.,* 2020). Multiple studies have shown the consequences of microplastic ingestion during early development, delaying emergence, reducing growth, and altering body shape (Martinez-Gomez *et al*., 2017; Messinetti *et al.,* 2018; Lo & Chan, 2018).

The variability of plastic particle size and density stratifies their accumulation in the environment e.g. in sediments, throughout the water column, and the surfaces of waterways and oceans, which in turn affects their portability (Thompson *et al.,* 2004; Barnes *et al.,* 2009). For example, low density particles are subject to stronger surface wind and wave movement due to this increased buoyancy, whilst high density particles settle in benthic sediments (Barnes *et al.,* 2009). This stratification of plastics provides low to medium density particles an increased probability of greater movement from their point of origin through various hydrodynamic processes (Hidalgo-Ruz *et al.,* 2012; Thompson, 2015). This is problematic because particles act as vectors of pollutants e.g. hydrophobic organic contaminants (HOCs), and toxic trace elements (Cole *et al*., 2011; Li *et al.,* 2017).

Plastic production also requires a range of additives, plasticisers, and stabilisers which often contain endocrine disrupting compounds (e.g. bisphenol and phthalates), and heavy metals (e.g. chromium and cadmium) which can leach into the environment, or surrounding tissues when ingested (Li *et al.,* 2017). Furthermore, HOCs present in the environment (e.g. DDTs, PCBs) have the capacity to adsorb onto the surface of microplastics, and subsequently desorb when ingested by wildlife (Bakir *et al*., 2014; Bradney *et al.,* 2019). In crustaceans, microplastics have been shown to leach toxic chemical elements that interfere with the production/reception of crustacean hyperglycaemic hormones (CCH) (e.g. moult inhibiting hormone). CCHs are responsible for a wide range of biological processes in crustaceans such as the regulation of hemolymph glucose, and the moult cycle (ecdysone synthesis), and as a homeostatic control mediator in the stress response (Chung, 1999; Böcking & Dircksen, 2002; Kim *et al*., 2013). Fluctuations in CCH have also have the capacity to interfere with energy availability, its investment in essential behaviours, and consequently metabolic functions such as camouflage (Fanjul-Moles, 2006; Kim *et al*., 2013).

Marine Environmental Warming

Climate change is frequently thought of as a future issue, with emission targets either pushed back or entirely unmet (Falkner, 2016). However, the effects of climate change are already evident from decreased sea-ice coverage, to rising sea levels, and perturbed weather patterns, all of which impact the marine environment, and have the

potential to affect aquatic biodiversity (Craig, 2012; Doney *et al.,* 2012). One of the primary consequences of climate change is ocean acidification through CO_2 sequestration, which subsequently leads to increasing ocean temperatures (Doney *et al.,* 2009). Anthropogenic climate change is not only expected to affect the mean thermal conditions of the marine environment, but also increase the frequency of extreme thermal events (e.g. heatwaves and El Niños) (Schulte *et al.,* 2011). It is believed that these extreme thermal events may be more important in predicting species' future spatial distributions than gradual environmental trends (Harley & Paine, 2009).

Marine invertebrates display a wide range of thermal sensitivities and tolerance limits, with hardier species exhibiting a broader range of thermal tolerances and reduced sensitivity to environmental fluctuations (Doudoroff, 1945). However, all species experience thermal stress when exposed to temperatures that approach or exceed their upper thermal limits (Whiteley & Mackenzie, 2016). The variation in thermal tolerance and broad capacity to compensate for environmental change, is thought to be related to the spatial environment from which a species originates e.g. relative latitude, or vertical distribution along the shore, as well as their life stage (Whiteley & Mackenzie, 2016). However, many species are specifically adapted to their thermal environment, and therefore may fail to thrive outside of their particular thermal niche. This is especially true of sessile invertebrates who are unable to escape temperatures that exceed their thermal optima by moving to more favourable microclimates (Somero, 2002). Research by Gunderson and Stillman (2015) suggests that despite aquatic taxa generally possessing greater plasticity than their terrestrial counterparts, ectotherms exhibit low thermal tolerance acclimation, and therefore the risk of overheating will remain for even the most plastic of species.

One of the main functions impeded in ectothermic species when exposed to temperatures approaching their thermal minimum or maximum, is metabolic enzymatic activity (Kern *et al.,* 2015). Proteins function over a narrow range of temperatures and are susceptible to denaturation though misfolding or entirely unfolding, as such they are particularly susceptible to fluctuations in the local thermal environment. When subjected to short-term exposure such as flash heatwaves, individuals are able to recover by producing protective molecules such as heat shock proteins and antioxidants (Whiteley & Mackenzie, 2016). However, long-term

exposure to such temperatures is known to affect fitness and survival by exceeding the temperatures necessary for reproduction and growth (Pörtner, 2010). Furthermore, long-term exposure has been linked to decreased phenotypic plasticity, particularly an individual's capacity to acclimate thermally, which may also hold consequences for antipredator phenotypic changes (Schulte *et al.,* 2011). Heat shock has been found to inhibit behavioural responses to predation in aphids (*Myzus persicae*) (Sentis *et al.,* 2017).

Multiple Stressors

To understand how multiple stressors impact marine ecosystems, we need to know not only how individual species will be affected, but also the interactions between individuals (Woodward *et al.,* 2010). Through understanding these interactions, we can better predict how they translate into outcomes at the population, and community level. This is particularly challenging as community dynamics, stressors, and species–stressor interactions frequently vary with season, environmental conditions, and intensity of disturbance (Lenihan *et al.,* 2018). Additionally, their combined effects cannot be accurately predicted using existing single-stressor studies, and short-term monitoring (Christensen *et al.,* 2006). Most organisms are able to exhibit some level of resilience when faced with a singular anthropogenically derived stressor (Lirman & Manzello, 2009; Hughes *et al.,* 2017; Rankin *et al.,* 2019). However, environmental stressors rarely exist in isolation within a natural setting and frequently act synergistically, increasing the severity of effects incurred through exposure and subsequently decreasing the probability of coping with additional stressors (Lange & Marshall, 2017).

Adaptation to environmental changes (e.g. climate change or pollutants) is greatly influenced by a species' plasticity, their potential for dispersal, and the latitudinal ranges they can inhabit (Bernhardt & Leslie, 2013). The intensity of abiotic stressors experienced by species can be influenced by the environment and season within which they arise, as this can affect whether they occur in or out of phase with one another. Thus, some organisms may be exposed to multiple stressors simultaneously, whereas others will experience them sequentially (Gunderson *et al.,* 2015). The energy required to overcome or mitigate each subsequent stressor rises with increasing stress intensity (Sokolova *et al.,* 2012). However, when one or more

environmental factor deviates from the individual's optimum and cannot be mitigated, it can lead to physiological disturbance, instigating a stress response that over time decreases fitness (Hoffmann & Parsons, 1994). This is supported by the life-history theory, which predicts that an increase in resource allocation (e.g. energy) to one process (e.g. thermal regulation to reduce thermal stress), decreases the resources available for allocation to other such processes (Stearns, 1992).

Ocean temperatures are a major determinant of local marine biodiversity, driving seasonal migration, the timing of biological events (e.g. breeding), and changes in food source abundance (Pörtner & Farrell, 2008). Many of these operate across narrow temperature ranges, and are therefore sensitive to changes in thermal composition. The long-term consequences of such broad fluctuations in temperature are unknown, but it is likely that they will significantly affect the global distribution of aquatic life (Craig, 2012). Should the current rate of climate change continue to increase, future environmental temperatures will exceed the current tolerances of many marine organisms, with little scope to adapt (Doney *et al.,* 2012). Therefore, environmental temperature should be considered among the most important and pervasive of abiotic factors. In ectotherms particularly, fluctuations in environmental temperature have the capacity to influence physiological functions due to the effects of thermodynamics on the biochemical reactions that underlie growth, reproduction, and many antipredator behaviours (Kern *et al.,* 2015; Mynott, 2019).

Environmental warming also impacts marine ecosystems through synergistic interactions with existing stressors, such as habitat destruction, overfishing, and marine pollution (Sokolova, 2013). Plastic pollution, with particular reference to microplastics, is a similarly pervasive stressor within the marine environment. While microplastics do have a tendency to aggregate, there is often broad geographical and spatial overlap between microplastic pollution and areas of increased environmental warming (Horton & Barnes, 2020). Temperature increases in the thermal environment have been found to increase the effects of toxicity following microplastic ingestion in *Daphnia magna* and *Daphnia pulex* (Jaikumar *et al.,* 2018). Kratina *et al.* (2019) suggest that exposure to higher environmental temperatures intensify the negative impacts of secondary microplastic ingestion on respiration rate further. Reduced respiration over an extended period has been shown to affect ATP production, and subsequent energy allocation. Under predicted future climate conditions, the

synergistic interactions between ocean warming and acidification may become more intense, causing amplified microplastic toxicity across marine invertebrate species (Bertucci & Bellas 2021). This makes their interactions as stressors pertinent to current marine conservation research.

Camouflage in a Changing World

While much is known about the use and optimisation of camouflage, our understanding of how anthropogenic activity may affect this potentially energetically costly process is limited (Talloen *et al*., 2004). Direct human disturbance in the marine environment such as dredging and shipping have been shown to affect escape responses in invertebrate species (Jenkins & Brand, 2001; Wale *et al.,* 2013; Wale *et al*., 2015; Carter *et al.,* 2020). Carter *et al.* (2020) also found that exposure to shipping noise induces stress in juvenile shore crabs (*Carcinus maenas*), subsequently reducing their background matching capacity. This is further compounded by the presence of chemical and plastic pollutants which can alter the biotic (e.g. predator composition) and abiotic (e.g.the decreased heat absorbance and retention properties of sediments) factors of marine environments (Carson *et al*., 2011; Fischer *et al*., 2013).

Changes in the abiotic conditions can also be indirectly affected by human activity, such as anthropogenic driven climate change. Much of our understanding of this topic is based around 21 terrestrial species that depend on seasonal coat colour moulting for camouflage (Zimova *et al.,* 2018). In the northern hemisphere climate change has led to an increase in ambient temperature, causing decreases in the duration of snowpack cover and leading to high-contrast mismatches between species colouration and their environment. This renders prey species such as the willow ptarmigan (*Lagopus lagopus*) at a greater risk of predation, causing severe implications for predator-prey dynamics (Zimova *et al.,* 2016; Zimova *et al.,* 2018). Phenotypic changes in colouration also serve other roles, such as UV protection and thermoregulation.

As climate change also leads to increasing UV-radiation, this may result in conflicting pressures between camouflage and UV/heat exposure for ectothermic species (Roulin, 2014). For example, dark colouration provides better UV-protection, however

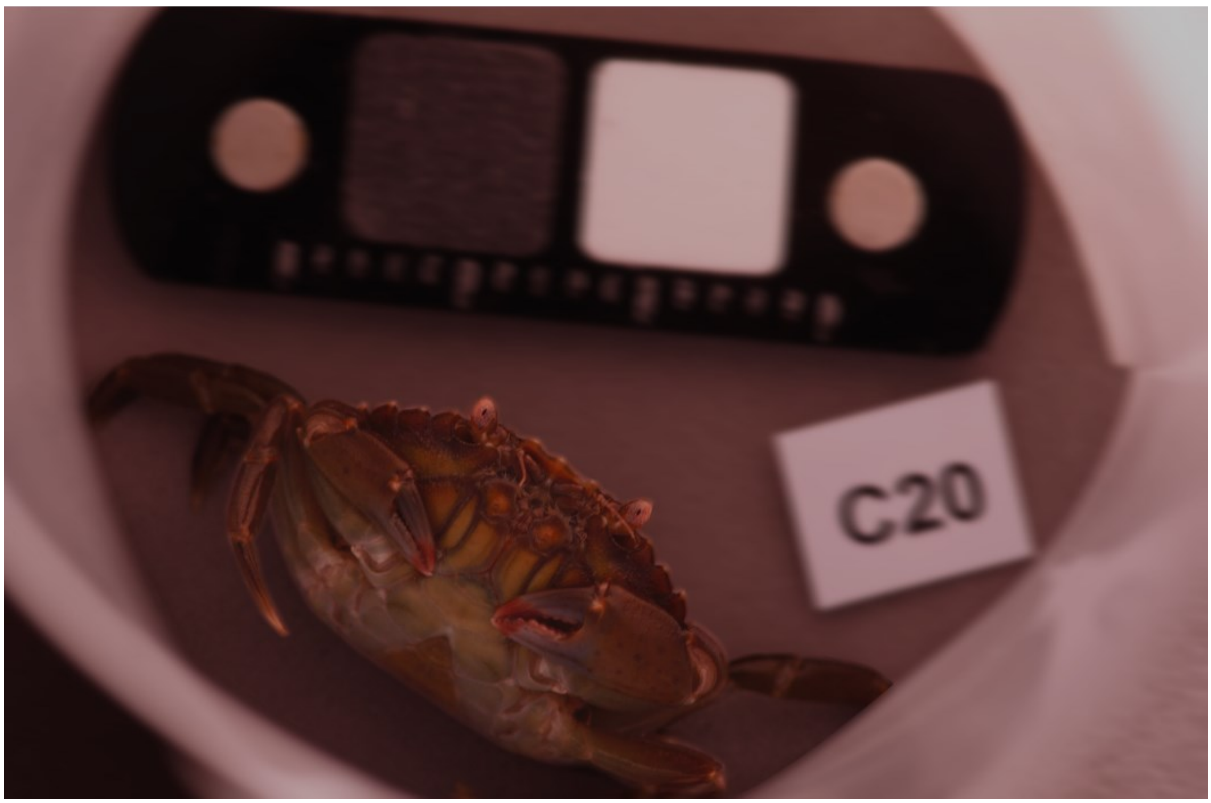
darker individuals that rely on the environment to aid with thermoregulation will be more liable to overheating (Roulin, 2014). Environmental warming caused by climate change has the capacity to alter oceanic currents that may in turn influence the future distribution and abundance of microplastics (Welden & Lusher, 2017). Understanding how the interactive and cumulative effects of multiple stressors impact anti-predator strategies, and by extension predator-prey interactions, is vital in predicting how an ecosystem may respond to future environmental change (Woodward *et al.,* 2010).

Thesis Purpose and Aims

Climate change and plastic pollution at present are considered to be among the greatest threats to marine biodiversity (Craig, 2012), and rank among the top 5 emerging issues for global conservation (Sutherland *et al.,* 2019). Despite the increasing public awareness of these issues, and the growing body of research behind them, research into the effects of anthropogenic stressors on behaviour and antipredator mechanisms have been largely neglected or overlooked. Instead focussing on the broadscale changes these stressors can induce e.g. species range shifts (Thuiller, 2004; Mitchell *et al*., 2006; Harley & Paine, 2009), or specific physiological aspects (e.g. body condition, fecundity, embryonic development) (Pörtner & Farrel, 2008; Somero, 2010; Wright *et al.*, 2013). Carter *et al.'s* (2020) findings of exposure to shipping noise significantly affecting camouflage and escape behaviour indicate that the effects of anthropogenically derived stressors on antipredator behaviour warrant further investigation. Furthermore, the existence of environmental stressors in isolation is rare within a natural setting, often co-occurring and acting synergistically to increase the severity of adverse effects. This thesis investigates the effects of microplastic ingestion as a single and combined stressor alongside environmental warming on juvenile shore crab survival. Specifically, I focused on the energetic consequences of these stressors on camouflage and growth; both as short, and long-term survival proxies. Few examine the effects on invasive invertebrate species, or the antipredator mechanisms that many of these species share. Additionally, there is a distinct lack of research into the effects of such stressors on intermediate juvenile stages. Thus, their study is vital in predicting how responses vary from a species to an ecosystem level.

Using juvenile shore crabs (*C. maenas*) as a model species, this thesis addresses some of these knowledge gaps through a series of long-term stressor exposure experiments in a laboratory setting. As an intertidal species, juvenile shore crabs are at the crux of plastic pollution sources and extreme environmental temperature gradients. Shore crabs are an abundant species globally, and play an important role in northern hemisphere food webs (Watts *et al.,* 2015). Through studying their response to common marine stressors, it allows us to deduce how survival mechanisms and antipredator behaviours may be impacted in other marine invertebrates. In Chapter 2 this thesis explores the energetic consequences of microplastic ingestion and retention on camouflage and growth; both as short, and long-term survival proxies. In Chapter 3, I focused on the effects of combined stressors (microplastic ingestion and environmental warming) on camouflage and growth. This was achieved by exposing shore crabs to two different temperatures (14 and 24°C), and feed with or without microplastics. In doing so, I directly explore how each stressor affects physiological and morphological traits, as well as how these stressors interact (e.g. synergistically or antagonistically). In order to maximise ecological validity, the use of digital image analysis was employed across both experiments to quantify changes in luminance using the vision of a relevant predator of shore crabs as in Stevens (2007), and Troscianko and Stevens (2015). The concentrations of microplastics used were considered to be ecologically relevant at the time of publication, and are reflective of higher particle abundances recorded in the North Sea and Danube River (Dubaish & Liebezeit, 2013; Lechner *et al.,* 2014). In the final chapter, we discuss the implications of these findings both in terms of short-term trade-offs for juvenile shore crabs, and long-term consequences at the population and ecosystem levels. I also suggest areas of future research to further close the remaining knowledge gaps, and better inform marine conservation policies.

Chapter 2: The effect of microplastic ingestion on *Carcinus maenas* camouflage and growth



Abstract

Microplastics are among the most prevalent forms of anthropogenic pollution, with far reaching consequences for many species through ingestion and internal accumulation. While many studies have outlined their associated effects on marine invertebrate physiology (e.g. reduced body condition and growth). The impact of microplastics on fundamental processes such as phenotypic plasticity, have yet to determined. Here, I investigate the effects of chronic microplastic ingestion on luminance (perceived lightness) change and subsequent background matching in juvenile shore crabs (*Carcinus maenas*). In a tank-based experiment, environmentally relevant concentrations of virgin microplastics (approximately 734 x 10^3 m^{-3}) were administered through feed on a weekly basis. Luminance change was induced by housing dark crabs on a white background for eight weeks. Image analysis using avian predator vision was used to assess luminance change and subsequent camouflage. Carapace width, and individual weight were also recorded as measures of growth. Our results show no observable differences in luminance change, or background matching

between treatment groups at either two or eight weeks. Furthermore, the rate of moulting, carapace growth, and weight change did not appear to be significantly affected by microplastic ingestion or retention. Our results did however find that the process of moulting may be used as a mechanism to rapidly clear microplastic burden. These findings suggest that low-level chronic exposure does not have direct consequences for juvenile growth, or camouflage in crustaceans, at least in isolation. However, this study does highlight that other species may be at risk if they are unable to successfully clear accumulated microplastics.

Introduction

Microplastics (1µm to 5mm in diameter) are considered to be the most predominant form of plastic pollution in the marine environment (Thompson *et al*., 2004; Conkle *et al*., 2018), accounting for around 92% of debris found at the ocean surface (Eriksen *et al*., 2014). Polyethylene, polypropylene, and polyvinylchloride being amongst the most commonly sampled in freshwater and marine environments (Li *et al*., 2017; Conkle *et al*., 2018; Lindeque *et al*., 2020). Coastal environments are believed to be a hotspot of microplastic generation and accumulation (Andrady, 2011), as they act as a buffer between marine and terrestrial environments. The variability of microplastic particle size and composition stratifies their accumulation in the environment e.g. in sediments, throughout the water column, and the surfaces of waterways and oceans (Thompson *et al*., 2004; Barnes *et al*., 2009). Particles that occur on the ocean surfaces are more liable to coastal deposition due to stronger wind and wave action (Hinata *et al.,* 2017). Furthermore, coastal habitats are at additional risk due to their close proximity to primary sources (e.g. roads and landfills) (Setälä *et al.,* 2016). As such, coastal habitats and their resident wildlife are considered to be among the most vulnerable.

The diversity of microplastic shape, colour and size increases the risk of ingestion by marine species, both directly and through trophic transfer (Bradney *et al*., 2019). The colour and shape of microplastics are thought to increase bioavailability through resembling prey items, particularly in visual predators such as seabirds (Wright *et al*., 2013). Once ingested, the detrimental effects of microplastics may be mechanical (e.g. blocking of the digestive tract), energetic (hindering growth and mobility), or chemical (interfering with endocrine processes) (Setälä *et al*., 2016). The production of plastic

requires a large range of additives, plasticisers, and stabilisers. These frequently contain endocrine disrupting compounds (e.g. bisphenol and phthalates), and heavy metals (e.g. chromium and cadmium), which can leach into the environment and surrounding tissues when ingested (Li *et al*., 2017). Low levels of chronic exposure are generally not considered to be lethal. However, they have been shown to significantly reduce feeding, and result in the depletion of energy stores (Wright *et al*., 2013; Cole *et al*., 2015). Particles <300μm have also shown high potential for bioaccumulation, and biomagnification in aquatic food chains as they are readily retained within organism tissues (Watts *et al*., 2014; Ferreira *et al*., 2019). Due to the widespread distribution and persistence of microplastics in the environment, it is imperative the scope of its effects on marine fauna is adequately researched. To date, there has been little research into the wider consequences of microplastic ingestion in marine invertebrates beyond basic functions such as fecundity, body condition, and growth (Wright *et al*., 2013; Cole *et al*., 2015; Galloway *et al*., 2017). Whilst these functions are essential to all species, many species maintain additional costly behaviours. One such behaviour that is fundamental to the survival of many marine species, is camouflage.

Background matching is among the most prevalent of camouflage strategies. It aims to minimise the appearance of feature variation between an animal and its chosen surroundings to avoid detection and recognition by visual predators (Merilaita & Stevens, 2011). Successful camouflage typically involves the process of phenotype matching to the individual's given environment. In background matching the phenotypes in question are generally hue, brightness, colour, pattern, and texture (Thayer, 1986; Nokelainen *et al*., 2017; Michalis *et al*., 2017). Cephalopod species are particularly adept at making dramatic changes in appearance, often incorporating colour, hue, luminance, and texture within the span of a few seconds (Hanlon *et al*., 2008). However, the ability to change colour slowly, over periods ranging from hours to weeks is considered to be a more common phenomenon in the natural world (Stevens *et al*., 2014; Duarte *et al*. 2017).

One such example of slower colour change are horned ghost crabs (*Ocypode ceratophthalmus*), which exhibit a circadian rhythm of colour change over a 24h period. This cyclical camouflage causes them to appear lighter during the day, gradually becoming darker at night by altering their colour and brightness to match

that of their substrate (Stevens *et al*., 2013). Similarly, the common shore crab (*Carcinus maenas*) utilises changes in brightness (luminance) to match their environment. Juvenile shore crabs are highly variable in appearance, and capable of small plastic changes over the course of several hours, to larger changes over a period of a number of weeks (Stevens *et al*., 2014, Carter *et al.,* 2020). This is thought to be due to juveniles possessing weaker physical defences than their adult counterparts when faced with an array of predators including other invertebrates, fish, and shore birds (Crothers, 1968).

The process of camouflage itself is generally regarded as energetically demanding (Talloen, *et al*., 2004; Bergstrom *et al*., 2012; Rogers *et al*., 2013). This is particularly true of juvenile shore crabs which attain the largest changes in appearance by adjusting the distribution of black and white pigments within chromatophore cells when moulting (Stevens *et al*., 2014; Stevens, 2016). This process involves balancing the growth of new tissues, up/down regulation of pigments, and general metabolic functions with respect to their energy budgets (Duarte *et al*., 2017). Typically, this is achieved through the intake of energy (food) and minimising energy loss (reducing unnecessary movement). To maximise energy intake, shore crabs display opportunistic feeding habits, preying on a range of smaller marine invertebrates, scavenging larger carrion, and occasionally participating in cannibalism (Rangeley & Thomas, 1987; Baeta *et al*., 2006).

Variation in feeding, however, provides multiple avenues for microplastic debris to become ingested orally (primary ingestion). Shore crabs, as their name suggests, are typically found in coastal and estuarine intertidal environments (Crothers, 1966). The highest concentrations of microplastic particles are also frequently observed in such coastal and estuarine environments (Claessens *et al*., 2011; Desforges *et al*., 2014; Auta *et al*., 2017). Watts *et al*., (2014) demonstrated that shore crabs are liable to secondary inspiration of microplastics across their gills in an aqueous environment. In both cases microplastics Watts *et al*. (2014) found microplastics to be retained in either the foregut or the gill surface, with microplastics on the gills persisting after 21 days of exposure. Microplastics have a significant negative effect on branchial function, food consumption, and significantly reduce available energy (Watts *et al*., 2015; Watts *et al*., 2016). However, these effects are transient and decrease following the cessation of exposure (Watts *et al*., 2016). In their natural environment where microplastic

exposure is constant, this persistence of particles within organs and bodily tissues could have more permanent effects than observed in a laboratory setting. Therefore, it is likely this will have knock-on effects for other energetically costly behaviours such as conspecific interaction, fleeing predation, and camouflage. Due to the overlap in pervasiveness of both camouflage and microplastics, it is likely that the effects and subsequent implications are not limited to shore crabs.

This chapter explores how microplastic ingestion impacts luminance change in juvenile shore crabs, as perceived by an ecologically relevant predator. I address the relationships between camouflage, growth, and primary/secondary virgin microplastic ingestion. This experiment was undertaken as a long-term laboratory study, with weekly measurements of carapace colouration and growth (carapace diameter, and weight). Juvenile shore crabs were housed on white backgrounds to induce luminance change over a period of eight-weeks. Microplastics were administered via their feed (processed gelatinous mussel cubes) at a concentration of approximately 734 x 10^3 m^{-3} on a weekly basis. Luminance change and camouflage efficacy was analysed using avian predator vision on multispectral images. We hypothesise that chronic exposure to ecologically relevant levels of microplastics will reduce an individual's capacity to acquire energy, and subsequently deplete existing energy reserves. In doing so, this may reduce the available energy for processes such as luminance change. Therefore, decreasing the overall efficacy of background matching, leaving individuals more liable to predation. Furthermore, a reduction in energy intake or existing energy stores is likely to also have consequences for juvenile growth, with reduced carapace size and no net weight gain per moult. Alternatively, there could be no detectable impact on luminance change and camouflage, or juvenile growth (weight/carapace). This would imply that shore crabs (and other crustaceans) may be able to tolerate low-level microplastic ingestion and retention through mitigation of possible detrimental effects.

Methods

Ethical Note

Experimental research was conducted with the approval of the Biosciences ethical committee of the University of Exeter (application ID 2018/2317). Due to the nature of

the experiment, all individuals used were unable to be returned to their point of origin due to the risk of releasing microplastic contaminants into the environment, and as such were euthanised in accordance with RSPCA recommendations in lieu of equivalent marine invertebrate ASPA regulations. Shore crabs are not considered endangered or protected, and therefore no additional licences were required to carry out this research.

Procedure overview

Wild-caught juvenile shore crabs (*Carcinus maenas*) were kept under laboratory conditions to assess the effects of primary microplastic ingestion and secondary inspiration on camouflage. Crabs were housed on white backgrounds for a period of eight weeks as outlined in Mynott (2019) and Carter et al., (2020), during which they were fed weekly under one of two treatments: control or microplastic spiked, adapted from Watts *et al.* (2014). Individuals were also photographed and measured on a weekly basis in case an insufficient number of individuals survived the full eight weeks.

Resulting images were analysed to assess the effects of microplastic ingestion and retention on the extent of luminance change, and the efficacy of the individual's overall background matching. This was achieved through the use of model predator vision, using methodology developed by Troscianko and Stevens (2015). Following the experiment, all remaining individuals were euthanised and dissected for microplastic tissue analysis. Tissue processing protocol was modified from Hermsen *et al.,* (2018), and analysis based on Watts *et al.* (2014).

Study Species and Collection

88 juvenile shore crabs (<30mm carapace diameter) were collected from the upper intertidal zone at Gyllyngvase Beach (50°08'32.5"N 5°04'09.3"W), Falmouth, UK during the summer of 2018. Crabs were collected within 3 hours either side of low tide. Individuals were selected based on size (approximately 15-25mm carapace diameter). This is because the most notable changes in colour occur in juveniles, due to increased cuticular thickness and calcium carbonate deposition in individuals exceeding 25mm as they reach sexual maturity (Powell, 1962b; Crothers, 1968; Baeta, 2005). Owing to the variability of the rockpool habitat juveniles were found in, some patterning and mixed colouration was present. Sex was not taken into account during collection as it cannot be reliably determined in juveniles (Mohamedeen &

Hartnoll, 1990). Collected crabs were placed into grey buckets with fresh seawater, rocks, and algal cover to reduce stress, before being transported back to a laboratory-based animal facility at the University of Exeter, Penryn Campus, UK. Following their arrival, individuals were assigned to treatment groups according to carapace diameter, colour, and patterning, so as to ensure an even distribution between treatment groups.

Figure 1.1: Variable camouflage within juvenile shore crabs. A) Juvenile shore crab exhibiting bright colouration and contrast patterning consistent with disruptive camouflage in complex environments. B) A dark, uniformly coloured individual from the same environment that was used in the luminance change and background matching experiments.

Tank set-up and husbandry

Treatment groups (control and microplastic) were housed in two separate tanks (one per treatment) to prevent control group contamination. Each tank measured 90 x 44.5 x 30cm, and was segmented using UV-transmitting Perspex to ensure individual separation, reducing the risk of cannibalism and agonism (Figure 1.2). The base of each compartment was lined with waterproof paper (black or white) and contained a 2cm depth of gravel (Swell Harlequin Gravel, Swell UK, Cheshire, UK). The addition of a particulate substrate provides a naturalistic environment that permits burying behaviour and reduces individual stress (Chabenat *et al.,* 2019).

Crabs were kept for 1 week prior to the start of the experiment to acclimatise to the following aquarium conditions: 15°C, 12:12h day/night cycle, artificial seawater (35ppt). This also allowed for the partial expulsion of any current microplastics that may have been present in their digestive tract or gill surfaces.

Each tank contained 150L of recirculating, dechlorinated artificial saltwater (Instant Ocean, Blacksburg, Virginia). Tanks were fitted with external filters (Classic 350 filter;

Eheim GmbH & Co., Deizisau, Germany) with the capacity to filter approximately 620L/hour. Filter inflow and outflow hosing were positioned at opposite ends of each tank. Inflow hosing was placed directly into the base of an unoccupied compartment, a matrix of small holes at the base of each compartment were drilled to allow waste waterflow with minimal obstruction. Outflow hosing was attached to a suspended pipe network above the tank, each pipe contained small perforations to allow aerated water to be deposited evenly into compartments. Temperature was set to the average sea temperature at the time of specimen collection (15°C), and regulated using a DC300 Aquarium Chiller (The Aquarium Solution Ltd., Ilford, UK). Salinity and temperature were subject to daily monitoring, with weekly water quality testing to ensure they were within safe parameters (NH3+ <0.25mg/L, NO2- <0.3mg/L, NO3- <0.2mg/L , pH = 8) (Yusoff *et al*., 2011). A 12:12 hour day/night cycle was used, beginning at 7:00 and ending at 19:00 (TMC GroBeam Ultima Strip 'natural daylight'; AquaRay, Hertfordshire, UK). Black gravel was used during the acclimatisation period to reduce the likelihood of carapace colour change, followed by white gravel for the experimental phase.

Tanks were cleaned on a weekly basis while crabs were removed for feeding. The cleaning regiment consisted of removing any algal deposition, a 50% water change, clean gravel substitution, and removing visible faecal deposits. Tanks were given a 'deep clean' – full water removal and replacement, and removal of algae/faecal/microplastic traces. Conducted mid-way through the experimental phase to facilitate an exchange between treatment groups to account for any tank effects.

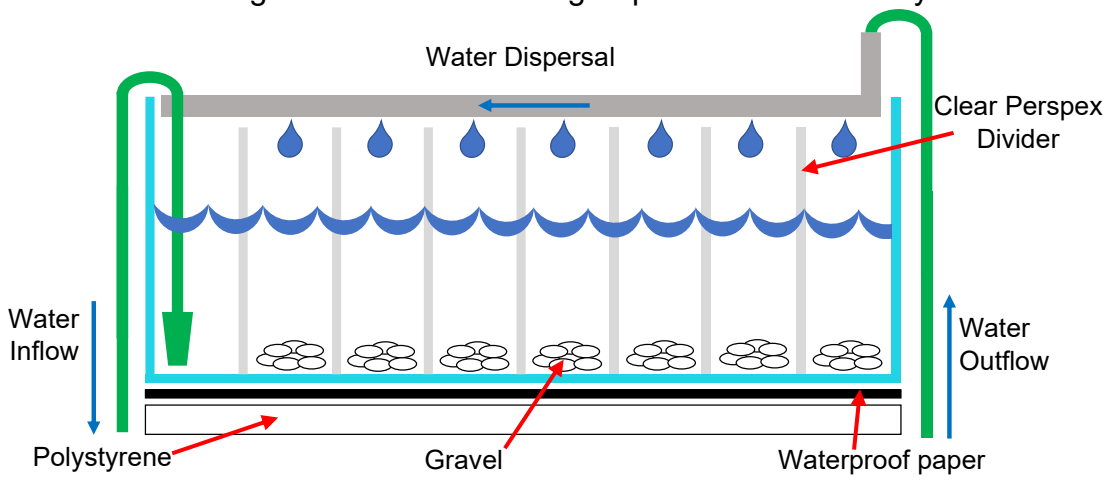


Figure 1.2: Experimental set-up for experiment 1. Cross section diagram of the housing tank set-up used during the experiment. A matrix of holes at the base of each compartment divider allowed for water to circulate within the tank.

Microplastic Master Mix

Four common plastic pollutants were used to create the microplastic master mix: Polyamide (PA), Polyvinylchloride (PVC), Polyethylene Terephthalate (PET), Polyhydroxy-Butyrate (PHB) in equal parts measured by weight (Figure 1.3). The microplastics chosen for use reflects their persistence in the environment, the frequency of their use in industry, and increased availability. The microplastics used were sourced from Dr Andrew Watts at the University of Exeter, having been measured and fluorescently labelled prior to the commencement of this project. To achieve the desired microplastic size range for the master mix, some plastics (e.g. Polyamide) which are less frequently found in particle surveys than other polymers (e.g. Polyethylene), were included. Microplastics were created by mechanically fragmenting macroplastics using a coffee bean grinder to obtain a range of different particle sizes. These were then fluorescently labelled using RADGLO (475nm) (Radiant Color NV, Houthalen, Belgium), to aid identification of microplastics within specimen tissues (Figure 1.3), and after processing. Area, mean particle diameter, and particle count per gram were recorded prior to administering in feed (Table 1.3). The range of microplastic sizes and types used to compose the master mix reflect the composition of microplastic fragment pollutants commonly found in the marine environment (Conkle *et al*., 2017; Lindeque *et al*., 2020).

Experimental Feed

Jellified mussel feed composition was adapted from Watts *et al*. (2014), and consisted of 100g mussel (*Mytilus edulis*) tissue homogenate, 7g ground crustacean feed for maximal nutrition (Ocean Free Super Crustanorish sinking pellet), 13g of food-grade gelatine dissolved in 140ml distilled water (70°C for 30 min), with the addition of the microplastics master mix at a 0.5% concentration by weight to experimental feed. The mixture was then vortexed for 3 minutes, and pipetted into 1 x 1 x 1cm cube moulds. These were then set overnight at 5°C, and subsequently frozen at -18°C. Prior to use, cubes were thawed for 30 minutes and cut in half (0.45 ± 0.08g) to create individual portions. The final concentration of microplastics was 110,231 ± 389 particles per portion (based on the analysis of four replicate cubes).. Feeding was facilitated outside of the main holding tanks in 50ml containers containing 30ml of artificial seawater (Instant Ocean) at 15°C for six hours. This was to ensure all feed had been consumed

by each individual. The maximal microplastic exposure per feed was approximately 734 x 10^3 m^{-3}, which is reflective of higher particle abundances recorded in the aquatic environment, such as those by Lechner *et al.,* (2014) and Dubaish & Liebezeit (2013). However, due to transfer between feeding containers and holding tanks following consumption, loss of particles to the water column during feeding etc. the final concentration will be considerably lower. Shore crabs exhibit short periods of no feeding activity around moulting (Adelung, 1971), so those that had moulted directly prior to or during feeding were fed 24 hours later.

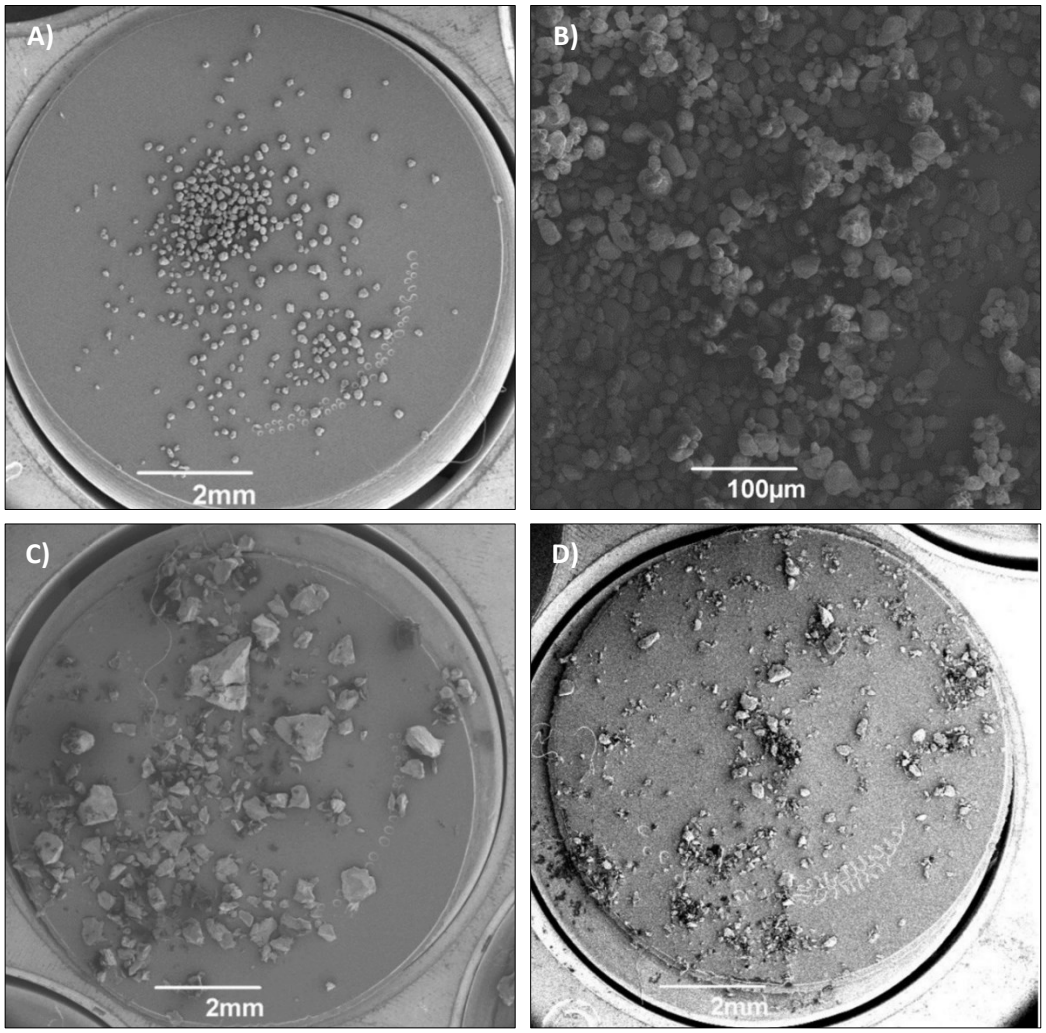


Figure 1.3: Scanning electron microscope images of microplastics used in master mix post grinding. A) Polyvinyl Chloride (PVC). B) Polyamide (Nylon). C) Polyethylene Terephthalate (PET). D) Polyhydroxy-Butyrate (PHB). Images courtesy of Dr Andrew Watts.

Table 1.1: Microplastic master mix particle dimensions. Particle diameter ranged from around 9-390 µm, covering a large proportion of microplastic sizes encountered within the marine environment. Measurements provided by Dr Andrew Watts as part of previous research by the University of Exeter.

Microplastic Master Mix		Area (μm^2)		Max Diameter (µm)		Min Diameter (µm)		Particle Count (/g)
Plastic	Abbreviation	Mean	SE	Mean	SE	Mean	SE	Particles
Polyvinyl Chloride	PVC	12.80	0.43	156.1	2.84	115.14	1.88	4222222
Polyamide (Nylon)	PA	1.38	0.10	50.28	1.30	35.1	1.43	360000000
Polyethylene Terephthalate	PET	82.62	9.94	390.64	18.03	247.13	12.65	365192
Polyhydroxy-Butyrate	PHB	19.39	1.41	198.25	6.97	122.45	3.89	1564554

Photography and Image Analysis

In preparation for photographing, each crab's carapace was cleaned using a soft bamboo toothbrush to remove excess algal deposition and blotted dry to reduce glare and spectral reflectance. In order to minimise stress, and therefore any subsequent short-term colour change (Powell, 1962a); individuals were measured and weighed after photographing and returned to their compartments within 20 minutes. To limit the risk of microplastic contamination, the control group was always photographed and measured first, using separate identical equipment where possible. Following this, crabs were transferred onto an intermediate grey foam surface, along with a black and white standard with 7% and 93% reflectance and scale to account for any variation in illumination over time. A translucent PTFE shield was placed between the crab and the light source to create a diffuse light environment. Photos were taken under controlled lighting conditions using a UV and human visible emitting Arc Lamp (70W 1.0A power source, Ventronic EYE Colour Arc Lamp, Venture Lighting Europe LTD., Watford, UK) equipped with a daylight 65 bulb to simulate natural daylight (Mynott, 2019; Carter *et al.,* 2020).

Photographs were recorded weekly between 8:30 am and 4pm to account for the natural circadian rhythm of shore crabs in which their carapace darkens during the course of the day and lightens again overnight (Powell, 1962b). Crabs that moulted less than 24h prior to their usual photograph day were photographed the following day, in order to allow the new carapace to harden, pigment to settle and prevent damage to the new exoskeleton during handling and cleaning.

Following the methodology outlined in Troscianko and Stevens (2015), photographs were taken using a full-spectrum sensitivity camera (Nikon D90 SLR, fitted with a 105mm Nikkor lens). This is achieved through the removal of the ultraviolet (UV) and infrared (IR) blocking filter (Advanced Camera Services LTD., Norfolk, UK). Camera aperture was kept constant, and white balance was set to manual. Two photographs were taken per individual per session, one using a UV/IR blocking filter allowing human spectrum visible only photographs (400-700 nm, Baader Venus U filter), and one UV pass IR blocking (300-400nm, Baader UV/IR cut filter). A custom filter slider was fitted to lens to allow images to be taken in quick succession. Reducing the possibility of subject movement between photographs.

All RAW image files were imported into ImageJ (version 1.8.0_112, National Institute of Health, NIH), and analysed using the Multispectral Image Calibration and Analysis Toolbox (Mica Toolbox version 1.22) developed by Troscianko & Stevens (2015). Images were first linearised to compensate for the nonlinearity of the camera's response to light intensity (Stevens *et al*., 2007), and then inspected to omit overexposed images from analysis. UV and visible images were overlaid to create a single multispectral image at each time point. Image pixel values were scaled so that a value of 65,535 on a 16-bit scale is equivalent to 100% reflectance, with images calibrated using the 93% and 7% reflectance standards. Following this, ROIs (regions of interest) of the carapace or gravel were selected from these multispectral images, avoiding areas of specular reflectance (where light reflects directly at the camera) that would prevent accurate analysis (Figure 1.4).

The extent of individual luminance change and subsequent camouflage was assessed in an ecologically relevant manner using the visual system of a common predator. In this instance, a model of peafowl (*Pavo cristatus*) vision was used to discriminate the level of background matching exhibited by shore crabs. Peafowl possess a visual sensitivity (violet-sensitive) similar to that of many shorebird species which are known predators of crustaceans (Crothers, 1968). Due to the experimental backgrounds being achromatic, carapace colouration (e.g. hue and saturation) were not analysed. ROI mean luminance (perceived object brightness) was calculated using the predicted double cone response, which is considered to underpin the achromatic vision of many bird species (Osorio & Vorobyev, 2005). This was achieved through converting multispectral images from camera colour space to a peafowl vision model using the

Batch Multispectral Analysis Tool (Troscianko & Stevens 2015). This mapping approach is regarded to be more accurate for modelling predicted photoreceptor stimulation in comparison to similar approaches that instead rely on reflectance (Stevens and Cuthill, 2006; Troscianko & Stevens, 2015). We were then able to calculate discriminability using the absolute difference between the crab and its background using the ROIs to determine the accuracy of background matching achieved, as seen in Stevens *et al*., 2013 and Carter *et al.,* 2020. A resulting low value would imply there is little discriminability between the two, and therefore suggest higher camouflage efficacy.

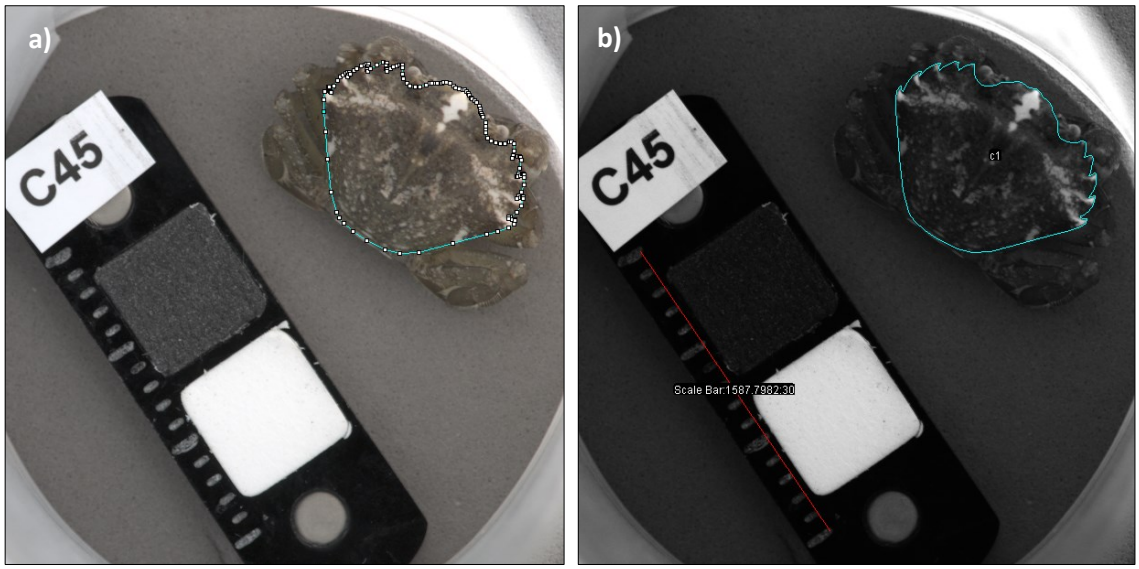


Figure 1.4: Image analysis using imageJ Batch Multispectral Analysis Tool. A) Region of Interest (ROI) selected under the Visual tab reflecting actual colouration of specimen. B) ROI selected under the aligned linear tab for analysis of carapace luminance. A multi-point ROI was used to capture the outline of the carapace as accurately as possible. Standardised shape coverage was omitted in favour of accuracy.

Tissue Processing and Analysis

At the end of the study period, crabs were transferred into 15ml containers, and put into a state of torpor at -18°C for 24 hours, followed by 24 hours at -80°C for euthanisation. A spike was then inserted through the ganglion to ensure all specimens were deceased prior to dissection. Each specimen's left gill and digestive tract were removed and weighed prior to freezing separately at -18°C for future processing. The

control group was also euthanised to check for microplastic contamination following the tank exchange midway through the experiment.

Tissue samples were processed using a solution of potassium hydroxide (Extra Pure Potassium Hydroxide SLR Pellets (Fisher Scientific, Loughborough, UK) at a 20% concentration for 48 hours at 60°C (Genlab LCO/9V/SS/GDIG/SMC, Widnes, UK) to dissolve the organic matter. This protocol was adapted from Hermsen *et al.,* 2018. Following this samples were rehydrated with 25ml of distilled water and vortexed for 30 seconds to resuspend microplastic particles. Five 20µL aliquots of each tissue sample were pipetted into individual wells of black UV 96-well plates, and microplastics were counted manually using a Leica DM IL LED inverted microscope. Control crabs were processed first to reduce the risk of microplastic contamination. Microplastic concentrations for the right gill were on average 20,000 (± 475) per individual, and 42,750 (± 675) in the digestive tract for those that did not moult over the whole experiment.

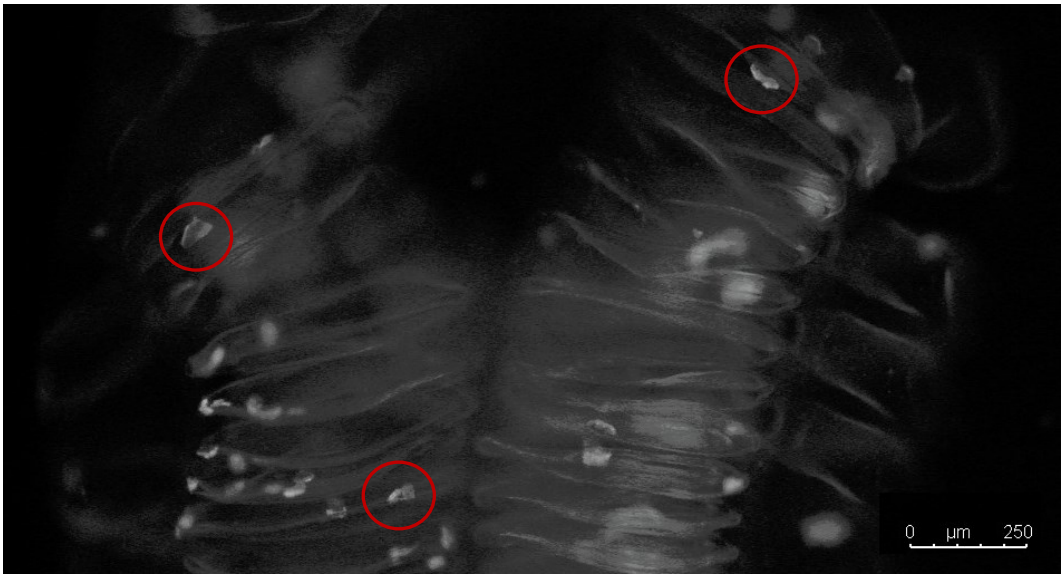


Figure 1.5: Microplastic fragments fluorescing on an illuminated gill segment. Fragments present through secondary exposure (inspiration). For ease of identification, some fragments have been highlighted in red above, but the actual quantity present is not limited to those highlighted. Image taken using a Leica DM IL LED inverted microscope.

Statistical Analysis

Of the original 88 shore crabs collected, 64 were included in the final statistical analyses (34 control, 30 microplastic). The majority of those omitted from statistical analysis were due to individuals becoming >25mm in diameter (carapace) during the

acclimatisation period. Carapace sizes exceeding 25mm indicate the individual has reached sexual maturity (adulthood), and therefore would exhibit reduced phenotypic plasticity. Several escaped their compartments overnight, meaning they were not exposed to the colour change inducing backgrounds in excess of 12h. A small number were targets of cannibalism despite best efforts to keep individuals separated.

Statistical analyses were carried out using R v.3.6.1 (R Core Team, 2019). The data generated from multispectral images in week two were preferentially used during statistical analysis over week one due to reduced stress levels experienced by individuals at this time point, providing greater accuracy of luminance change measurement. General Linear Models (GLMs) were used to test for the effects of microplastic ingestion on background matching, luminance change, and growth (weight and carapace diameter). The appropriate GLM family and link were determined using visual inspection of quantile-quantile plots, residual distributions, residual vs fitted value plots, and the skewness function (e1071 package v.1.7-3, TU Wien, Austria) to assess normality. Where changing GLM family was not appropriate data were transformed to reduce skewness. Information regarding changes to family, link, and data transformations are provided within the text. Maximal model controlling was used in conjunction with model simplification outlined by Crawley (2011) to produce the most parsimonious model per dataset. Variables controlled for included: tank, moulting, growth (weight and carapace diameter). The Akaike Information Criterion (AIC), Variance Inflation Factor (VIF), and overall model deviance were used to successively remove non-significant terms.

A GLM was not appropriate to analyse the time taken to moult by individuals in each group, instead a survival analysis in the form of a Cox proportional-hazard model (CPHM) was performed (Fox & Weisberg, 2011). CPHMs assess multiple factors simultaneously (e.g. treatment, size (carapace diameter), tank)) and how they affect the rate and probability of an event occurring (Cox, 1984). The minimum adequate model was determined by systematically removing non-significant terms. The ‘event’ term was set to the occurrence of an individual moulting, with ‘censored’ being assigned to individuals that did not moult within the duration of the experimental period. Hypothesis test statistics for both GLMs and CPHMs are presented within the results section.

Results

The mean luminance of individuals at the end of the acclimatisation period (beginning of the experiment phase) was not found to significantly differ between treatment groups (Kruskal Wallis, $\chi^2_{(1)}$ =2.18, p=0.14).

Week Two

Luminance Change

Microplastic ingestion did not significantly affect the extent of luminance change after the first week of exposure (GLM, $\chi^2_{(1,62)}$ =<0.001, p=0.85). Size and moult were controlled for in the model, both of which were found to significantly affect luminance change (Size: GLM, $\chi^2_{(1,60)}$ =0.009, p=0.003; Moult: GLM, $\chi^2_{(1,61)}$ =0.005, p=0.02), with individuals that had moulted exhibiting greater changes in luminance than their non-moult counterparts (Figure 1.6). An interaction between treatment and moult was also included (GLM, $\chi^2_{(1,58)}$ =<0.001, p=0.46), but subsequently removed as it did not significantly affect the model's AIC or deviance, as was tank (GLM, $\chi^2_{(1,59)}$ = <0.001, p=0.98).

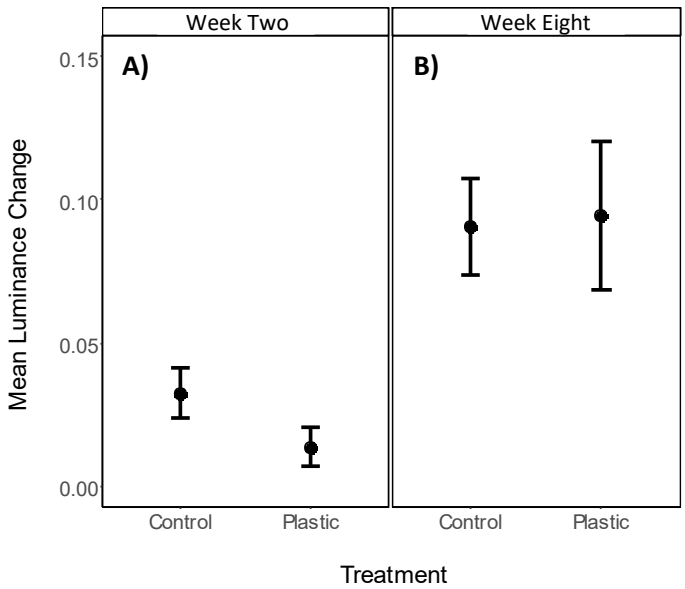


Figure 1.6: Mean luminance change between treatment groups after A) two weeks, B) eight weeks of exposure to experimental feed and colour change inducing backgrounds.

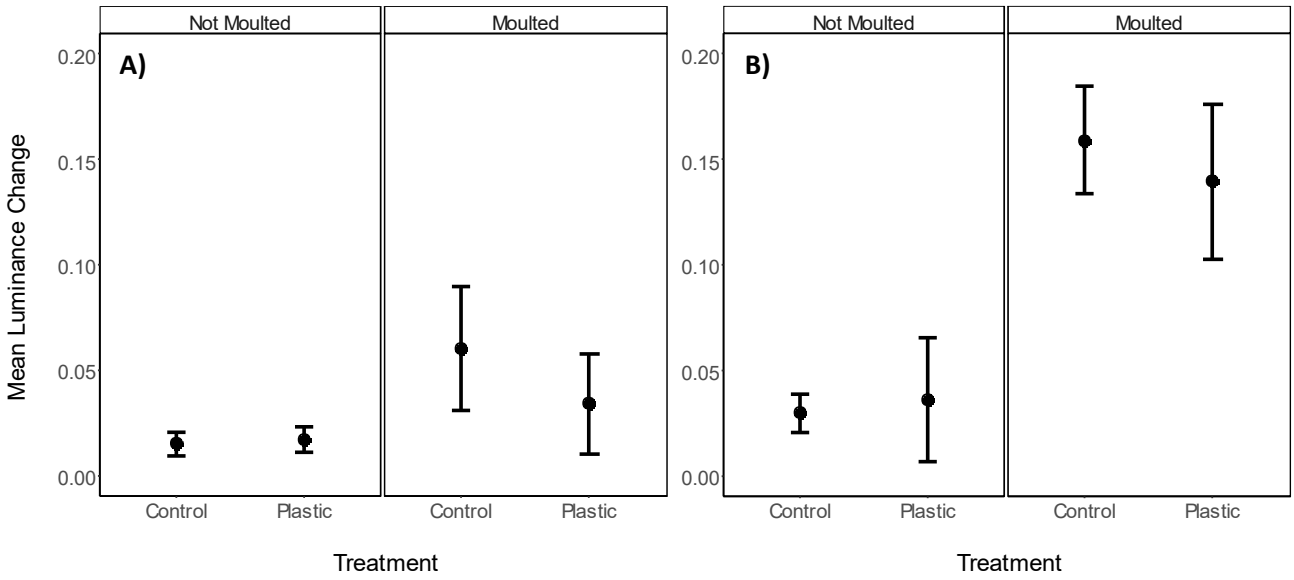


Figure 1.7: Mean luminance change between individuals that did and did not moult. A) after two weeks of exposure n=9. B) after eight weeks of exposure n=33. The raw values of the absolute difference between two given weeks were used.

Background Matching

Raw background matching data presented with a minor negative skew, a Gamma family GLM with inverse link was used to correct for this. The degree of background matching displayed by individuals after two weeks of exposure was not found to be significantly affected by microplastic ingestion (GLM, $\chi^2_{(1,62)}$ =0.01, p=<0.13) (Figure 1.8 A). Moulting and size were controlled for in the model and found to both significantly affect background matching (Moult: GLM, $\chi^2_{(1,61)}$=0.06, p=<0.001; Size: GLM, $\chi^2_{(1,60)}$ =<0.02, p=0.44), with those that had moulted matching their new experimental background to a greater extent. Tank, and an interaction between treatment and size were also initially included, but removed as both did not significantly affect the model's AIC or deviance (Tank: GLM, $\chi^2_{(1,59)}$ =<0.001, p=0.68; Treatment + Size: GLM, $\chi^2_{(1,58)}$ =<0.001, p=0.74).

Growth

Weight change data showed a strong positive skew but also contained negative values (weight loss), prohibiting the use of another GLM family. Raw weight change data was therefore cube root transformed to meet the normality assumptions of the Gaussian GLM family. Weight change over the first two weeks of the experiment was not significantly affected by microplastic exposure (GLM, $\chi^2_{(1,62)}$ =0.41, p=0.1). Moult, size (carapace), and tank were controlled for in the model, with only moult significantly

affecting any changes in weight (GLM, $\chi^2_{(1,61)}$ =6.22, p=<0.001). Tank, and size did not significantly affect weight change or the model's deviance/AIC, and were therefore removed (Tank: GLM, $\chi^2_{(1,59)}$ =0.06, p=0.51; Size: GLM, $\chi^2_{(1,60)}$ =0.05, p=0.57). An interaction between treatment and size was included and subsequently removed as it did not significantly affect weight change (GLM, $\chi^2_{(1,58)}$ =0.25, p=0.21), or the model's overall deviance.

Changes in carapace diameter were not analysed after two weeks as only 9 crabs had moulted by this time point as carapace diameter is fixed between moults. Therefore, we would not expect to see a change in carapace diameter when no moult had occurred, resulting in an exceedingly small sample size. Carapace growth across the entire length of the experiments was consequently analysed under the subheading of 'Growth', in 'Week Eight'.

Week Eight

Luminance Change

Raw luminance change data showed a minor positive skew but included negative values preventing the use of another GLM family, as such was cube root transformed to meet the normality assumptions required when using GLMs. Microplastic ingestion did not significantly affect the luminance change exhibited by individuals between week two and eight of the experiment (GLM, $\chi^2_{(1,62)}$ =0.28, p=0.06). Size, tank, and moult were initially controlled for in the model, with only moult found to significantly affect luminance change (Size: GLM, $\chi^2_{(1,60)}$ =0.001, p=0.88; Tank: GLM, $\chi^2_{(1,59)}$ =0.05, p=0.4; Moult: GLM, $\chi^2_{(1,61)}$ =1.3, p=<0.001) (Figure 1.7 B). Size and tank were removed from the model as they did not significantly affect the model's deviance or AIC. There was also no significant interaction between treatment and moult (GLM, $\chi^2_{(1,58)}$ =0.04, p=0.49). However, this variable was subsequently removed as it did not significantly affect the model's deviance.

Background Matching

Due to the strong negative skew of the raw background matching data, a square root transformation was performed to meet the normality assumptions of GLMs. The extent of background matching displayed by individuals after eight weeks of exposure was not found to be significantly affected by microplastic ingestion (GLM, $\chi^2_{(1,62)}$ =<0.001, p=<0.7) (Figure 1.8 B). Moulting and size were controlled for in the model (Moult: GLM,

$\chi^2_{(1,61)}$ =0.11, p=<0.001; Size: GLM, $\chi^2_{(1,60)}$ =0.006, p=0.3) however, only moult was found to significantly affect background matching. Size was subsequently removed as it did not significantly affect the model's deviance or AIC. Tank, and an interaction between treatment and size were also initially included, both were removed as they did not significantly affect the model's AIC or deviance (Tank: GLM, $\chi^2_{(1,59)}$ =0.003, p=0.48; Treatment + Size: GLM, $\chi^2_{(1,58)}$ =0.002, p=0.55).

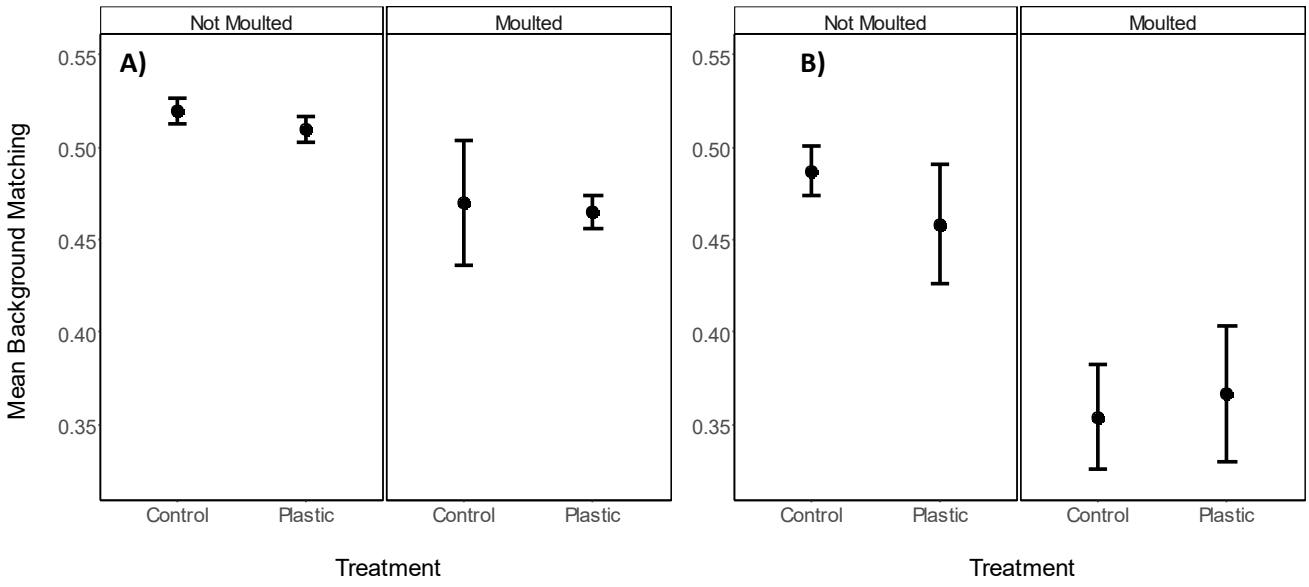


Figure 1.8: Mean background matching: A) after two weeks of exposure – those that had moulted n=9. B) after eight weeks of exposure – those that had moulted n=33. The absolute difference between the crab and background, between two given time points were used.

Growth

Microplastic ingestion did not have a significant effect on the weight change of individuals across the eight weeks of the experiment (GLM, $\chi^2_{(1,62)}$ =1.31, p=0.21). Moulting, size, and tank were controlled for in the experiment initially, with moult and size significantly affecting weight change (Moult: GLM, $\chi^2_{(1,61)}$ =23.69, p=<0.001; Size: GLM, $\chi^2_{(1,60)}$ =38.93, p=<0.001). Tank was not found to be significant and was subsequently removed as it did not have a substantial impact on the AIC or deviance of the model (GLM, $\chi^2_{(1,59)}$ =2.90, p=0.60). An interaction between treatment and size was initially included, and then removed as it did not significantly affect the model's deviance (GLM, $\chi^2_{(1,58)}$ =0.14, p=0.67). After reviewing Figure 1.9. B), we also analysed the weight change specifically in those that did not moult as it is suggestive

of a difference between treatment groups. However, this was also not found to be significant (GLM, $\chi^2_{(1,29)}$ =3.07, p=0.06).

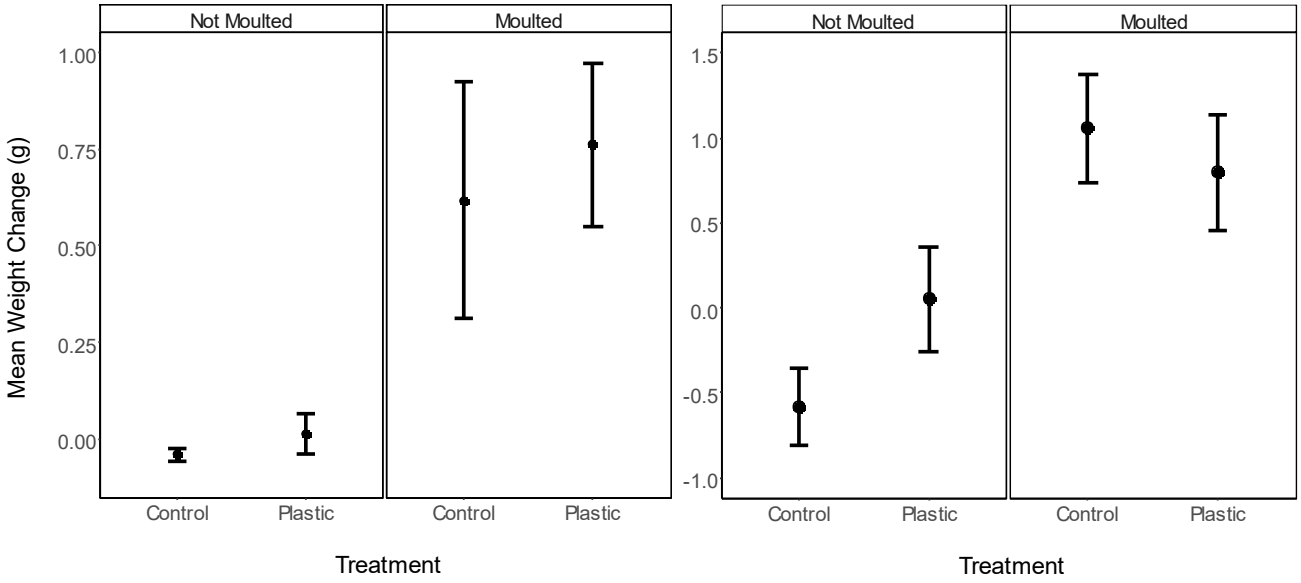


Figure 1.9: Mean weight change. A) after two weeks of exposure (moults n=9). B) after eight weeks of exposure (moults n=33). The observed scale of change used for A) is much smaller than in week eight. The control group in B) exhibited greater weight loss than their plastic treatment counterparts, although this was not found to be significant.

The changes in carapace diameter of those who moulted were not found to be significantly affected by microplastic ingestion (GLM, $\chi^2_{(1,31)}$ =0.001, p=<0.97). Start size and tank were included but also not found to be significant, however did affect the deviance and AIC of the model and so were not removed (Start: GLM, $\chi^2_{(1,30)}$ =0.91, p=0.34; Tank: GLM, $\chi^2_{(1,29)}$ =0.22, p=0.64). Only those that moulted were initially used for this as carapace diameter is fixed between moults. However, following the above results we repeated the GLM using the results of all crabs to confirm that moult was indeed the only variable that affected carapace growth (GLM, $\chi^2_{(1,61)}$ =192.23, p=<0.001).

Moult

Microplastic ingestion did not significantly affect the rate or probability of moulting during the course of the eight weeks (Cox Proportional Hazard, $\chi^2_{(1)}$ =0.56, p=0.45) (Figure 1.10 B). An effect size (hazard ratio) of 0.98 was calculated for microplastic ingestion. An effect size value close to one suggests that there is no difference between the rate of moulting expressed between treatment groups. Tank was initially

included as a variable; but was subsequently removed as it did not significantly improve the model's deviance (Cox Proportional Hazard $\chi^2_{(1)}$ =0.01, p=0.89).

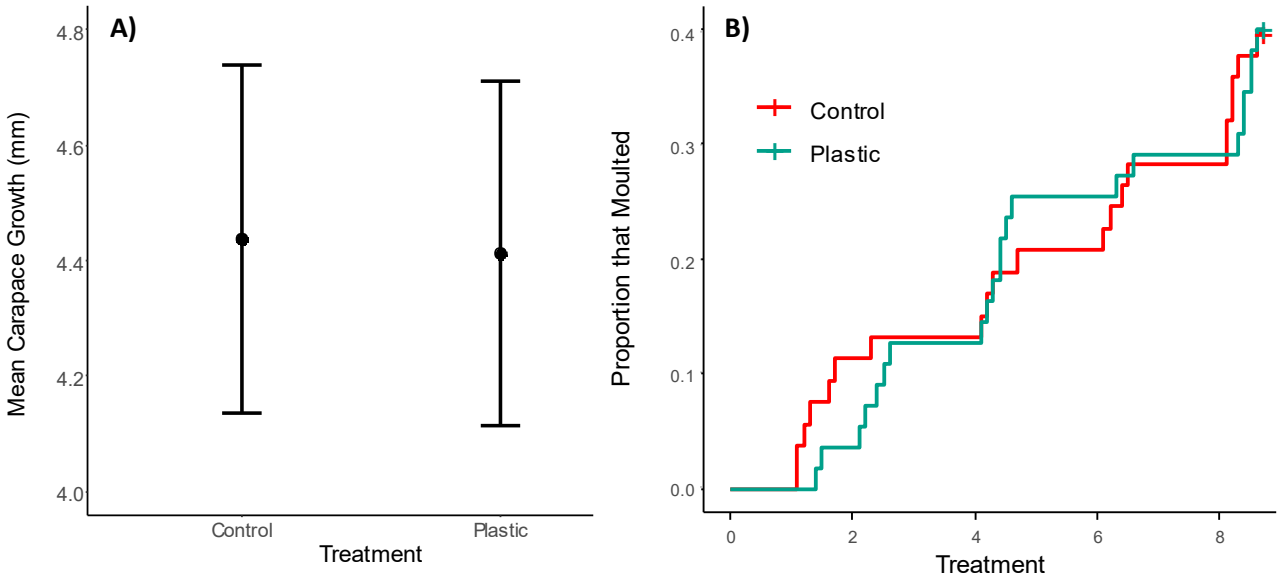


Figure 1.10: Mean carapace growth, and the proportion of individuals that had moulted. A) Mean carapace growth over the whole experimental period of crabs that moulted. B) The proportion of crabs that had moulted at each time point (day), over the course of the experiment. Those that had moulted n=33 (control = 16, plastic = 17). A '+' at the end indicates individuals who had not moulted by the end of week eight.

Microplastic Loading

The overall mean microplastic load was significantly different between individuals who had moulted, and those who had not during the course of the experiment (Kruskal Wallis: $\chi^2_{(1)}$ =62.82, p=<0.001). Individuals who had not moulted possessed on average 31,750 ± 1,844 more particles, than those that had. The high standard error reflects the variability of miroplastic loading due to individuals moulting at different times, and subsequently shedding their load. Those that moulted in the last week were not included in this calculation as they would not have recieved microplastic spiked feed prior to the end of the experiment.

Discussion

Low-level chronic exposure to microplastic did not reveal any measurable morphological, or physiological changes in juvenile shore crabs. There was no

significant difference observed between treatment groups with regards to luminance change at any point during the experiment. As such, background matching was also unaffected by primary and secondary microplastic ingestion. This indicates that microplastic ingestion is unlikely to affect the plethora of marine organisms that utilise background matching and other forms of energetically costly camouflage (see Chapter 3). This finding also suggests that in the short term there are no survival implications for species when solely looking at the effects on camouflage.

Virgin microplastics were used in this study which are defined as having the potential to leach unknown chemicals (e.g. additives), but have not been subjected to biofouling or hydrophobic organic contaminants (Martinez-Gomez *et al*., 2017). Additives frequently include the use of heavy metals such as cadmium, which have been linked to the inhibition of moulting through reducing ecdysone secretion, and by extension limiting growth (Moreno, 2003; Rodriguez *et al*., 2007). During this study there was no significant difference observed in carapace growth between treatment groups, or in the rate of moulting (Figure 1.10). This may indicate that the microplastics did not leach toxic chemical elements that interfere with the production/reception of the crustacean hyperglycaemic hormone (CCH) family (e.g. moult inhibiting hormone). The CCH family is responsible for a wide range of biological processes in crustaceans. For example: the regulation of hemolymph glucose, regulation of the moult cycle (ecdysone synthesis), and as a homeostatic control mediator in the stress response (Chung, 1999; Böcking & Dircksen, 2002; Kim *et al*., 2013). Fluctuations in CCH are recognized as having the capacity to interfere with energy availability, and consequently its investment in essential behaviours, and metabolic functions (Fanjul-Moles, 2006; Kim *et al*., 2013). Our findings suggest that low-level chronic exposure does not cause fluctuations in the secretion of CHH. Rather, it may change how metabolic energy stores are used in the short term without an immediate effect on the individual (Limonta *et al*., 2019). Therefore, allowing shore crabs to cope with the presence of microplastics. However, exposure to environmentally relevant concentrations of microplastics has also been shown to affect the expression of genes related to immunity and metabolic pathways (Limonta *et al*., 2019). Consequently, it is possible that the long-term effects of exposure may negatively affect how an organism responds to adversity (e.g. environmental stressors or pathogens) when energy reserves have been otherwise allocated (Lei *et al*., 2018; Limonta *et al*. 2019).

Growth with regards to weight change indicated no significant differences between treatment groups in the first two weeks of the study. A difference in weight change between weeks one and eight (Figure 1.8, B) showed a mean net loss within the control group, and net gain in the plastic group. Upon further analysis this was found to be non-significant (p=0.06), however the small sample size of those that did not moult (plastic n=13, control n=18) could be limiting to the analysis. It is possible that microplastic loading over an extended period of time could yield significant differences in weight change. However, it is unknown whether this would be detrimental to the individual's immediate condition or pose long-term implications for survival. Furthermore, given the small quantities of microplastics administered during the experiment, it is unlikely that the net gain observed in non-moult treatment crabs would be caused by the weight of ingested particles alone.

Shore crabs primarily use two mechanisms to remove sequestered particles from their gills. Initially each gill chamber contains a flabella (gill rake), which sweeps across the gills using the setae to dislodge and push the particles towards the exhalent channel (Cavey *et al*., 1992). In addition to this, the scaphognathite (pumping organ) can reverse the flow of water in order to displace particles from the gills (McMahon & Wilkens, 1983). This study has highlighted is that the process of moulting may also serve as a strategy for removing microplastic particles from the gills. Decapod growth is achieved through moulting, which is the shedding of the existing exoskeleton to reveal a new larger exoskeleton underneath. During the process of ecdysis, the gill epithelium is shed along with the old exoskeleton. This process has previously been postulated as a method for removing sequestered materials such as heavy metals, and parasites from the gill surface (Martin *et al*., 2000). In juvenile shore crabs, moulting occurs every couple of weeks, slowly decreasing in frequency with age until around the 18th moult when they reach terminal ecdysis (Crothers, 1967). In this experiment we found a significant difference in microplastic load between those who had and had not moulted. An average of 20,000 (± 475) particles per non-moulted individual were found on the right gill, in comparison to only 5250 (± 250) in those that had moulted. Suggesting that moulting is a more effective method of removing sequestered microplastics, and thus negating any potential long-term effects of their presence. Watts *et al*., (2014), found that microplastics can persist on the surface of the gills over 21 days after initial exposure, with only a small portion removed through

other means. This may have severe implications for adult shore crabs who, in contrast to juveniles, moult once or twice a year or not at all if they have reached terminal ecdysis (Crothers, 1968). Consequently, adult shore crabs may experience a build-up of microplastics in their gills, reducing ventilation, and subsequently affecting basic metabolic functions as found by Watts *et al*., (2013; 2014; 2015). Adult shore crabs are also more likely to predate other species of invertebrates, younger conspecifics, and small fish in greater quantities. Exposing them to increasing amounts of microplastics in the process. Multiple studies have observed microplastic presence to be transient, suggesting a low biomagnification potential because of significant gut clearance (Watts *et al.*, 2014; Watts *et al.*, 2015; Güven *et al*., 2017). However, Watts *et al*. (2014) also found that, while microplastics are removed more readily from the digestive tract, particles were still detectable 14 weeks post exposure. Given more time, it is unclear whether individuals would continue to successfully mitigate microplastic ingestion/inhalation through constant low-level shedding of particles, and periodical moults. Additionally, these studies did not take into account smaller particles (<0.5μm) which have been shown to permeate into host tissues and hemolymph from the digestive tract (Farrell & Nelson, 2013). Therefore, retaining their high potential for bioaccumulation/biomagnification along marine food chains (Ferreira, *et al*., 2019).

The ubiquity of microplastics in marine environment (e.g. sediments, the water column, and ocean surface), means that particle exposure is likely to be a constant feature through all life stages of marine species. Multiple studies have shown the consequences of microplastic ingestion during early development (Martinez-Gomez *et al*., 2017; Messinetti *et al.*, 2018), delaying emergence, reducing growth, and altering body shape (Lo & Chan, 2018; Messinetti *et al*., 2018). This indicates that long-term rearing experiments of marine invertebrates through all developmental stages, could reveal how variations in concentration may have varying consequences for individuals later in life. This is particularly pertinent as there is contention as to whether the scientific community is underestimating micro- and nanoplastic abundance within the marine environment (Lindeque *et al*., 2020).

Although the incidence of microplastic ingestion and the associated deleterious physiological effects have been well documented in a range of marine taxa, many knowledge gaps still exist (Wagner & Reemtsma, 2019; Provencher *et al.,* 2019). Given that marine invertebrates account for over 60% of species diversity within the

marine environment (Ausubel *et al*., 2010), it is imperative that they continue to be the focus of many future studies. Marine invertebrates are a key component in the wider context of plastic pollution (Li *et al*., 2019; Macali & Bergami, 2020), due to their important role in food webs, as ecosystem engineers, and for their general contribution to the marine ecosystems (Leal *et al*., 2012; Nelms *et al*., 2018). The economic significance of marine invertebrate species should also not be understated, not only with regards to their contribution in the commercial fishing industry, but also as a source of new drug candidates (De Zoysa, 2012; Leal *et al*., 2012).

As this study was conducted solely under artificial conditions, care should be taken when extrapolating the results to a real-world setting as the abiotic conditions created differ to that of any given natural system. Primarily it is worth noting the differences in microplastic propagation both in an aqueous environment, and through dietary means. Microplastics in a small enclosed space such as a tank will behave differently to those in open water. Similarly, the ingestion and inhalation of microplastics would typically resemble constant low-level doses in a natural setting, rather than concentrated weekly doses. In addition to this, it is likely that microplastics were already present within individuals collected for study. Although a proportion of these particles will have been shed during acclimatisation, their presence at the beginning of the study could not be quantified without dissection. This study is perhaps among the first to explore how plastic pollution could directly impact camouflage, using ecologically relevant concentrations. It also demonstrates that despite an increasing anthropogenic presence in the marine environment, there is still hope that species may be able to mitigate and adapt to that the effects of plastic pollution. It is abundantly clear however, how vital it is that we continue to monitor sources of anthropogenic pollution, and further try to mitigate their far-reaching effects. Microplastic pollution is considered to be approaching ‘planetary boundary threat’ status, having already met the criteria of irreversibility and global ubiquity (Villarubia-Gomez *et al*., 2017). It is therefore not enough to simply regulate plastic manufacturing and disposal. As a species we need to actively move towards more sustainable, closed-loop, and biodegradable options to alleviate the immense pressure placed on fragile ecosystems.

Chapter 3: The effects of multiple stressors on 🦀🦀 *Carcinus maenas* camouflage and growth 🦀🦀

Abstract

Camouflage in the form of colour change is a common antipredator strategy employed by many taxa. While much is known about the use and optimisation of camouflage, there is limited knowledge on how it is influenced by anthropogenic activity. Few studies have assessed the effects of anthropogenically derived stressors (e.g. noise, climate change, plastic pollution) on phenotypic plasticity. Fewer still have assessed the cumulative effects of multiple stressors despite each rarely being present in isolation. This is particularly true of microplastic ingestion and climate change induced environmental warming. Here I investigate the effects of chronic microplastic ingestion and environmental warming on camouflage in juvenile shore crabs (*Carcinus maenas*). During this seven-week feeding study, juvenile shore crabs were exposed to virgin microplastics through feed (0.5% by feed weight), and environmental warming through a simulated heatwave (unseasonably high temperatures that may induce thermal stress), as single and combined stressors. Avian predator vision was used to discriminate the level of camouflage achieved in relation to a colour change inducing

background. Carapace width and individual weight were recorded as measures of growth. At two and six weeks of exposure, combined stressors did not influence camouflage when compared to microplastic ingestion and environmental warming alone. Exposure to multiple stressors did, however, significantly reduce weight gain and carapace growth compared to singular stressors. Those exposed to the higher temperature treatment (24°C) exhibited increased luminance (perceived lightness) change and subsequent camouflage in accordance with previous literature. These findings suggest that low-level chronic exposure to microplastic ingestion as a single or combined stressor does not have direct morphological consequences. However, there are physiological implications of exposure to combined stressors for juveniles, as camouflage may be maintained at the expense of growth, suggesting the presence of an energetic trade-off.

Introduction

Camouflage is considered the first line of defence for many species by reducing detectability and recognition of the individual to visually guided predators (Stevens & Merilaita, 2009; Troscianko *et al.,* 2013). While many camouflage strategies exist, they all typically rely on some combination of phenotypic, morphological, and behavioural adaptations (Stevens & Merilaita, 2009). Of these, background matching is thought to be among the most prevalent of camouflage strategies (Galloway *et al.,* 2020). Background matching entails altering the individual's phenotype to reflect colour and/or pattern of their given background in order to reduce salience. The diversity of forms found within this strategy is likely due to the vast array of backgrounds species encounter on a regular basis. Diversity in background appearance means that a fixed phenotype is unlikely to provide optimal camouflage against variable substrates, and strategies are needed to overcome this limitation (Stevens & Ruxton, 2019).

The ability to change colour is widespread among taxonomic groups, of which camouflage and sexual signalling are among the primary functions (Duarte *et al.,* 2017). Colour change for camouflage specifically entails the individual altering some aspect of their external phenotype with respect to hue, brightness, or colour to reflect those of their immediate surroundings (Thayer, 1986; Nokelainen *et al*., 2017; Michalis *et al.,* 2017). The mechanisms used for colour change generally fall into one of two

categories: morphological and physiological. Physiological changes typically occur faster (e.g. milliseconds to hours), whereas morphological changes are much slower, taking place over multiple days or weeks (Umbers *et al.,* 2014). In both cases, the process of colour change is assumed to be metabolically costly, requiring an increase in energetic expenditure, and thereby depleting the individual's overall energy reserves (Talloen *et al*., 2004; Bergstrom *et al*., 2012; Rogers *et al*., 2013). Behavioural adaptations (e.g. actively hiding under objects or burying) are often used in conjunction with colour change, and may temporarily offset metabolic costs (Stevens, 2016). However, if elevated energetic demands are not met through increased energy intake (e.g. through feeding) there may be prolonged physiological costs (e.g. reduced growth) (Rodgers *et al.,* 2013; Duarte *et al.,* 2017). Pigments typically associated with colour change also frequently serve alternative purposes in non-camouflage related functions (e.g. the immune response, UV protection, thermoregulation). As pigment creation is costly, any additional up regulation for camouflage represents a potential energetic trade-off with these key functions (Stevens & Merilaita, 2009; Stevens, 2016).

Environmental stress is known to negatively impact the conversion of energy within organisms, specifically redirecting energy allocation away from basal functions (e.g. growth and storage) towards those needed for immediate survival (Sokolova, 2013). Phenotypic plasticity is often regarded as central in the ability of organisms to respond to rapidly changing conditions and increased uncertainty (Donnelly, 2012). Ectothermic species are typically more vulnerable to rapid fluctuations in extreme temperatures, and may therefore experience severe performance decrements during a heat wave event when compared to endothermic species (Madeira *et al.,* 2018). The process of colour change in ectothermic organisms is strongly temperature dependent, with the rate of colour change reflecting a thermal response curve when exposed to increasing temperatures (Pörtner & Farrell, 2008). Furthermore, it has been found that early life stages in particular, have elevated sensitivity to environmental stressors (e.g. temperature, salinity, and chemical pollutants) (Sokolova *et al.,* 2012). This may be reflective of lower energy reserves found in juveniles, and the subsequent conflicting demands of development and mitigating stress (Parsons, 2003). Paganini *et al.,* (2014) show that increased environmental temperatures interact synergistically with low pH, inducing a physiological stress response in intertidal crab species, and

subsequently affecting their energy reserves. These tidal fluctuations in pH and temperature are further intensified by climate change, consequently heightening the stress response of individuals (Paganini *et al.,* 2014). Moreover, enzymes function over narrow range of temperatures, and are therefore susceptible to denaturation, as well as variation in activity relative to changes in the local thermal environment (Hochachka & Somero, 2002; Sokolova, 2013).

Research by Carter *et al.,* (2020) shows that exposure to anthropogenic stressors (e.g. noise pollution) can also induce physiological stress, constraining energy budgets and subsequently affecting the efficacy of antipredator behaviours such as camouflage. As previously mentioned, natural environmental fluctuations are common, however these are exacerbated by increasing anthropogenic activity which can act as a major source of environmental stress (Sokolova, 2013). The occurrence of extreme events associated with global warming (e.g. ocean heat waves) are set to continue increasing in frequency and severity (Mitchell *et al.,* 2006). When subjected to short-term exposure such as flash heatwaves, many invertebrate species are able to recover by producing protective molecules such as heat shock proteins and antioxidants (Whiteley & Mackenzie, 2016). However, long-term exposure to heightened temperatures has been shown to affect fitness and survival by exceeding the temperatures necessary for reproduction and growth (Pörtner, 2010). Long-term exposure has also been linked to a decrease in an individual's capacity to acclimate thermally (Schulte *et al.,* 2011). Given the rapid onset of these environmental changes, it is possible they may pose a threat to the survival of less resilient species if these changes are to persist in the long term (IPCC, 2013).

The ability to adapt to an increasingly changing environment is key to survival, although there are constraints to the degree of plasticity achieved. Hardy species such as shore crabs (*Carcinus maenas*) are able to adjust their physiology to mitigate stress and maintain adequate homeostasis in such environments. This leads to eventual population adaptation over multiple generations through individual acclimation (Madeira *et al.,* 2018). However, the process of acclimation is costly as the activation of the heat-shock response requires significant energetic investment due to the production of new proteins, and the repair/replacement of those damaged (Whiteley & Mackenzie, 2016). Consequently, acclimation may not always be optimal when faced with rapid onset environmental stress, resulting in energetic trade-offs and potential

physiological costs (Somero, 2002; Fitzgerald-Dehoog *et al.,* 2012). When environmental changes become physiologically intolerable, population migration is a common outcome. This results in the broad re-distribution of species and therefore is a key driver in marine biodiversity patterns (González-Ortegón *et al.,* 2013; Madeira, 2018). For those that cannot migrate or adapt, decreasing body condition, increased predation, and local extinction can follow (González-Ortegón *et al.,* 2013).

Unfortunately, environmental stressors are rarely present in isolation and their cumulative effects may be synergistic, additive, or even antagonistic (Crain *et al.,* 2008). As such, many species are increasingly inhabiting dynamic, and labile environments where several factors can change rapidly, and simultaneously. For example, microplastic pollution is a ubiquitous environmental stressor in the marine environment, and so is likely to coexist with other environmental stressors. Consequently, the already strained metabolic and physiological functions of marine species may be further impaired by microplastic ingestion and retention. Additives routinely used in the production of plastics (e.g. Bisphenol-A, phthalates, and brominated flame retardants) are known endocrine disruptors. These interfere with the development of the endocrine system and affect the functioning of organs that respond to hormonal signals (Campanale, 2020). Microplastic ingestion has also been linked to decreased efficiency in energy assimilation, and energy balance (Blarer & Burkhardt-Holm, 2016; Gardon *et al.,* 2018). This in turn reduces scope for activity, reproduction, and growth due to energy trade-offs between basal maintenance and other energy-requiring functions (Sokolova, 2012). However, the interactive effects of multiple stressors under environmentally realistic scenarios are not yet fully understood. Stressors typically have different physiological and molecular mechanisms that can interact in complex, non-linear ways. Therefore, the effects of combined stressors cannot be accurately predicted by examining the effects of a single stressor.

Understanding how the interactive and cumulative effects of multiple stressors impact anti-predator strategies, and by extension predator-prey interactions, is vital in predicting how an ecosystem may respond to future environmental change (Woodward *et al.,* 2010). This chapter explores how multiple stressors impact luminance (lightness) change in juvenile shore crabs, as perceived by an ecologically relevant predator. I address the relationships and trade-offs between camouflage and

growth, when under pressure from microplastic ingestion/retention and thermal stress. This experiment was undertaken as a long-term laboratory study, with weekly measurements of carapace colouration and growth (carapace diameter, and weight). Juvenile shore crabs were housed on white backgrounds to induce luminance change over a period of six-weeks. Individuals were equally divided into one of four treatment groups: control at 14°, microplastic at 14°C, control at 24°C, and microplastic at 24°C (combined ecological stressors). Microplastics were administered via feed (processed gelatinous mussel cubes) at a 0.5% by feed weight concentration (approximately 734 x 10^3 m^{-3} particles) on a weekly basis. Luminance change and camouflage efficacy was analysed using avian predator vision through modified multispectral images. We hypothesise that chronic exposure to ecologically relevant levels of microplastics and thermal stress will reduce an individual's capacity to acquire energy, and subsequently deplete existing energy reserves. In doing so, this may affect costly processes such as carapace luminance change to such an extent that it decreases the efficacy of background matching, leaving individuals more liable to predation. Furthermore, a reduction in energy intake or existing energy stores is likely to also have consequences for juvenile growth, with reduced carapace size, and reduced net weight gain per moult. Alternatively, if there is no detectable impact on luminance change or growth, individuals may be able to tolerate low-level microplastic ingestion and retention, or mitigate possible detrimental effects.

Methods

Ethical Note

Experimental research was conducted with the approval of the University of Exeter Biosciences Ethical Committee (application ID eCORN001661). As in Chapter 2, all remaining individuals were euthanised at the end of the experiment for tissue analysis. Euthanasia was conducted in accordance with RSPCA recommendations. Shore crabs are not endangered or protected, and therefore no additional licences were required to carry out this research.

Procedure overview

As in Chapter 2, wild-caught juvenile shore crabs (*Carcinus maenas*) were kept under laboratory conditions on white backgrounds for a study period of six weeks following protocols laid out in Stevens *et al.*, 2014 and Carter *et al.,* 2020. During this time crabs were exposed to one of two feeding treatments: control or microplastic (as in Watts *et al.,* 2014), and a further temperature treatment: 14°C or 24°C (adapted from Mynott 2019). Individuals were fed, measured, and photographed on a weekly basis to assess the effects of microplastic ingestion and thermal stress on the extent of carapace luminance change. Resulting images were analysed to assess camouflage efficacy as perceived by a model predator, using methods developed by Troscianko and Stevens (2015). Following the experiment, all remaining individuals were humanely euthanised following RSPCA guidelines, and dissected for microplastic tissue analysis. Tissue processing protocol was modified from Hermsen *et al.,* (2018), and tissue analysis based on Watts *et al.* (2014).

Study Species and Collection

88 juvenile shore crabs (<30mm carapace diameter) were collected from the estuarine mudflats at Penryn Quay (50°10'09.7"N 5°05'54.5"W), Penryn, UK between September 2018 and January 2019. Crabs were collected within two hours of low tide and stored in neutral grey buckets with rock and algal cover for transport back to the University of Exeter's Penryn Campus, UK. Individuals were selected based on size (approximately 15-25mm carapace diameter). This is because the most notable changes in colour occur in juveniles, due to increases in cuticular thickness and calcium carbonate deposition in sexually mature individuals (Powell, 1962b; Crothers, 1968; Baeta, 2005). Crabs were also selected based on uniformity of dark colouration. Following their arrival at the wet laboratory, individuals were assigned to treatment groups according to carapace diameter and colour. This was to ensure an even distribution of base size and colouration between each of the four treatment groups.

Tank set-up and husbandry

Treatment groups were housed in four separate tanks to prevent control group contamination. Each tank measured 90 x 44.5 x 30 cm, and was segmented using UV-transmitting Perspex to reduce the risk of cannibalism and agonism between conspecifics (Figure 2.1). The base of each compartment was lined with waterproof

paper (black or white), and contained a 2 cm depth of gravel (colour corresponding to waterproof paper) (Swell Harlequin Gravel, Swell UK, Cheshire, UK) to provide a naturalistic environment that permits burying behaviour and reduces individual stress (Chabenat *et al.,* 2019).

Crabs were kept for one week prior to the start of the experiment to acclimatise to the following aquarium conditions: 14°C/24°C, 12:12h day/night cycle, and artificial seawater (35ppt). 14°C is representative of average sea temperatures around the coast of Cornwall from late spring, through to summer and early autumn. Conversely 24°C represents the upper range of rockpool temperatures intertidal species may encounter during summer heatwaves. To reduce the possibility of thermal shock in the higher temperature treatments, tank ambient temperature was gradually increased by approximately 1.5°C daily, reaching 14°C /24°C at the beginning of the experiment (as in Mynott, 2019). This rate of temperature change is considerably slower than the daily changes that would occur due to tides and weather. The maximum temperature (24°C) experienced by individuals during this experiment did not exceed temperatures regularly experienced by shore crabs within the intertidal environment over acute exposures (Compton *et al.,* 2010). However, it does exceed those temperatures a shore crab may experience over an extended period at their native geographic southern range edge (coastal Mauritania), where sustained sea surface temperatures are typically between 19°C and 23°C (Zeeberg *et al.,* 2008). Given that the average sea surface temperature is expected to rise by 1-4°C by 2100 (IUCN, 2016), a chronic exposure of 24°C is a conservative representation of peak sustained sea surface temperatures at this geographical range as a result of climate change.

Each tank contained 150L of recirculating, dechlorinated artificial saltwater (Instant Ocean, Blacksburg, Virginia). Tanks were fitted with external filters (Classic 350 filter; Eheim GmbH & Co., Deizisau, Germany) with the capacity to filter approximately 620L/hour. Filter inflow and outflow hosing were positioned at opposite ends of each tank. Inflow hosing was placed directly into the base of an unoccupied compartment, a matrix of small holes at the base of each compartment were drilled to allow waste waterflow with minimal obstruction. Outflow hosing was attached to a suspended pipe network above the tank, each pipe contained small perforations to allow aerated water to be deposited evenly into compartments. Temperatures used were the average sea temperature at the time of specimen collection (14°C), and the upper thermal tolerance

for *C. maenas* (24°C) (Compton *et al.,* 2010). Temperature was regulated using a DC300 Aquarium Chiller (The Aquarium Solution Ltd., Ilford, UK), and two 50W underwater heaters (Superfish Nano Heater 50w; J&K Aquatics, Somerset, UK). Salinity and temperature were subject to daily monitoring, with weekly water quality testing to ensure they were within safe parameters (NH3+ <0.25mg/L, NO2- <0.3mg/L, NO3- <0.2mg/L , pH = 8) (Yusoff *et al.,* 2011). To compensate for evaporation, and an increase in salinity/pH during the course of the experiment, freshwater was added on an ad-hoc basis. A 12:12 hour day/night cycle was used, beginning at 7:00 and ending at 19:00 (TMC GroBeam Ultima Strip 'natural daylight'; AquaRay, Hertfordshire, UK). Black gravel was used during the acclimatisation period to reduce the likelihood of carapace colour change, followed by white gravel for the experimental phase to induce luminance change. See Chapter 2 for thorough outline of the tank cleaning regiment.

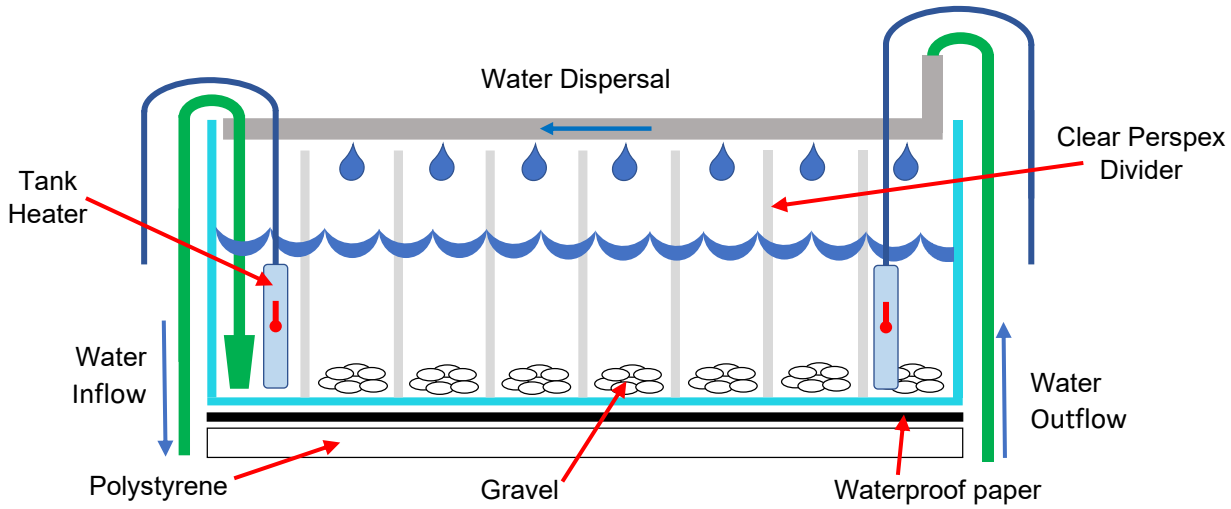


Figure 2.1: Experimental set-up for experiment 1. Cross section diagram of the housing tank set-up used during the experiment. A matrix of holes at the base of each compartment divider allowed for water to circulate within the tank.

Microplastic Master Mix

The same four common plastic pollutants that were used to create the microplastic master mix as in Chapter 2: Polyamide (PA), Polyvinylchloride (PVC), Polyethylene Terephthalate (PET), Polyhydroxy Butyrate (PHB), and were used in equal parts by weight. The microplastics used were sourced from Dr Andrew Watts at the University of Exeter. As in Chapter 2, microplastics were measured, fluorescently labelled, and mechanically ground to achieve a diverse size range prior to the start of the

experiments. The range of microplastic sizes and types used to compose the master mix reflect the composition of microplastic fragment pollutants commonly found in the marine environment (Conkle *et al.,* 2017; Lindeque *et al.,* 2020). Area, mean particle diameter, and particle count per gram were also recorded prior to administering in feed, please refer to Table 1.1 in Chapter 2 for details.

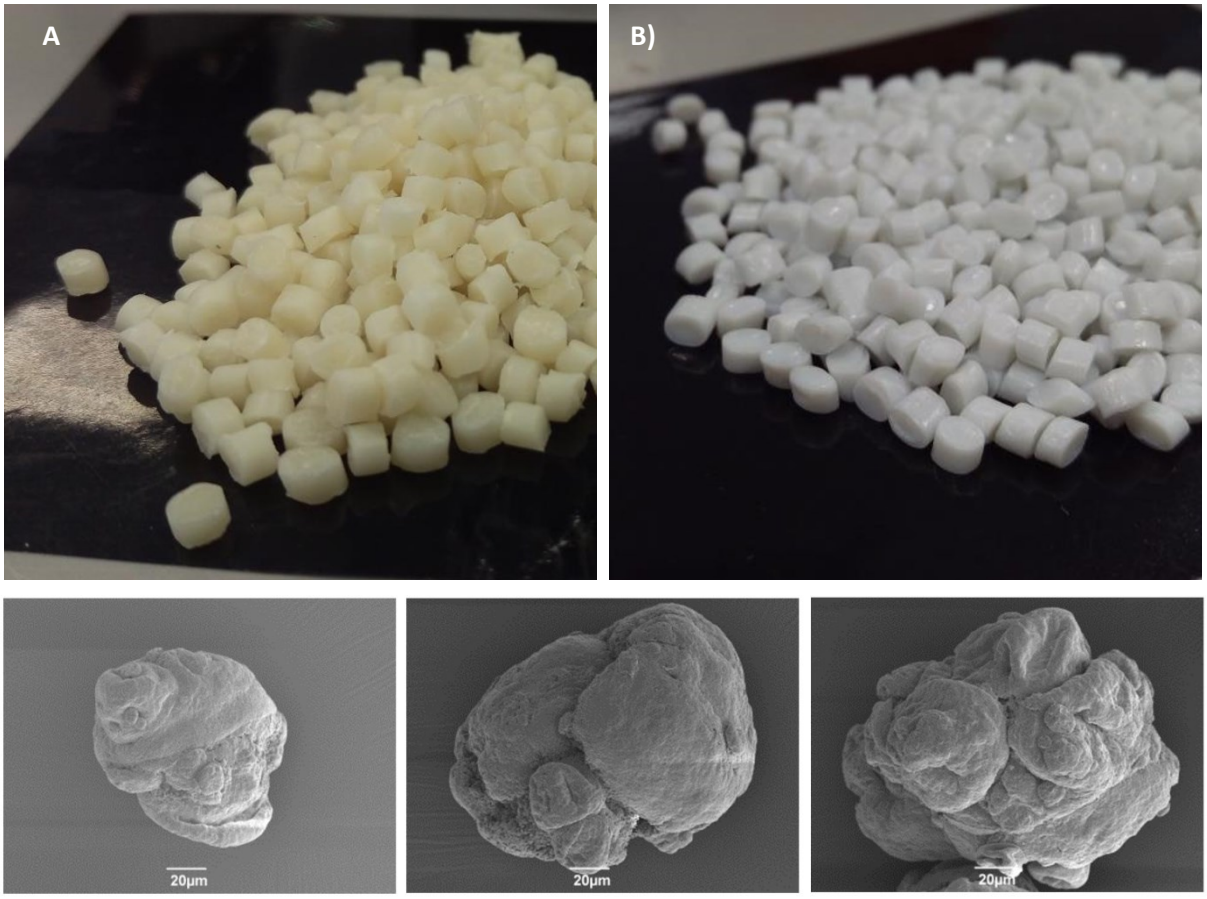


Figure 2.2: Nurdles (raw pre-production plastic pellets) A) Polyhydroxy Butyrate (PHB). B) Polyethylene Terephthalate (PET). C) Various PVC fragments obtained following the grinding process, demonstrating the variation in size and texture in particles used in the master mix. Each individual nurdle has a diameter of around 1cm. Images courtesy of Dr Andrew Watts.

Experimental Feed

Jellified mussel feed composition was adapted from Watts *et al.,* 2014, and consisted of 100g mussel tissue homogenate (*Mytillus edulis*), 7g ground crustacean feed for maximal nutrition (Ocean Free Super Crustanorish sinking pellet), 13g of food-grade gelatine dissolved in 140ml distilled water (70°C for 30 min), with the addition of the

microplastics master mix at a 0.5% concentration by weight to experimental feed. The mixture was vortexed for 3 minutes and pipetted into 1 x 1 x 1cm cube moulds. These were then set overnight at 5°C, and subsequently frozen at -18°C. Prior to use, cubes were thawed for 30 minutes and cut in half (0.45 ± 0.08g) to create individual portions. The final concentration of microplastics was 110,231 ± 389 particles per portion (based on the analysis of four replicate cubes). Feeding was facilitated outside of the main holding tanks in 50ml containers containing 30ml of artificial seawater (Instant Ocean) at temperatures corresponding to the crab's treatment group (14°C/24°C) for six hours. As with the previous experiment, this was to ensure all feed had been consumed by each individual (Figure 2.3). The maximal microplastic exposure per feed was approximately 734 x 10^3 m^{-3}, which is reflective of higher particle abundances recorded in aquatic environments, such as those in Lechner *et al.,* (2014) and Dubaish & Liebezeit (2013). However, due to transfer between feeding containers and holding tanks following consumption, loss of particles to the water column during feeding etc. the final concentration will be considerably lower. Shore crabs exhibit short periods of no feeding activity around moulting (Adelung, 1971), so those that had moulted directly prior to or during feeding, were fed 24 hours later.

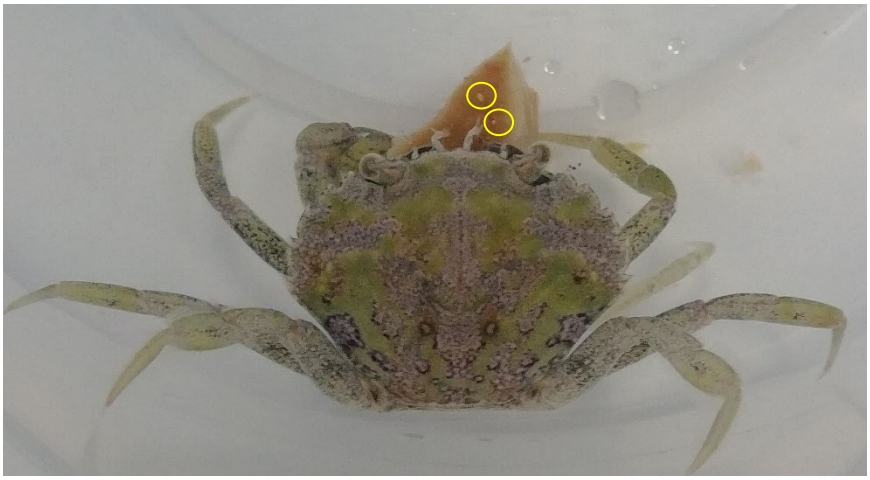


Figure 2.3: Juvenile shore crab feeding on weekly portion of experimental feed. Pictured feed contains microplastics, some larger fragments can be seen on the surface of the feed (circled yellow).

Photography and Image Analysis

Prior to photographing, each carapace was cleaned using a soft bamboo toothbrush to remove trace algal deposition, and blotted dry to reduce glare and spectral reflectance. Individuals were measured and weighed after photographing and returned

to their compartments within 20 minutes to minimise stress, and therefore any subsequent short-term colour change (Powell, 1962a). To limit the possibility of microplastic contamination, both control groups (control 14°C and control 24°C) were always photographed and measured first, using separate, identical equipment where possible. Following this, crabs were transferred onto an intermediate grey foam surface, along with a black (7%) and white (93%) reflectance standard and scale to account for changes in illumination between photographs. To create a diffuse light environment, a translucent PTFE shield was placed between the crab and the light source. Photographs were taken under controlled lighting conditions using a UV and human visible emitting Arc Lamp (70W 1.0A power source, Ventronic EYE Colour Arc Lamp, Venture Lighting Europe LTD., Watford, UK) equipped with a daylight 65 bulb to simulate natural daylight conditions (Mynott, 2019; Carter *et al.,* 2020). Photographs were recorded on a weekly, and during daylight hours to account for the natural circadian rhythm of shore crabs (Powell, 1962b). Individuals that moulted within 24h of scheduled photographing, were photographed the following day. This was to ensure the new carapace has ample time to harden to prevent damage, and for pigment to settle. Specific details of camera equipment used for photography can be found in Chapter 2.

All RAW image files were imported into ImageJ (version 1.8.0_112, National Institute of Health, NIH), and analysed using the Multispectral Image Calibration and Analysis Toolbox (Mica Toolbox version 1.22) developed by Troscianko & Stevens (2015). Images were first linearised to compensate for the nonlinearity of the camera's response to light intensity (Stevens *et al.,* 2007), and then inspected to omit overexposed images being included in analysis. Multispectral images of each time point were created by combining UV and visible images of each subject. Image pixel values were scaled so that value of 65,535 on a 16-bit scale is equivalent to 100% reflectance, with images calibrated using the 93% and 7% reflectance standards. Following this, ROIs (regions of interest) of the carapace or gravel were selected from these multispectral images, avoiding areas of specular reflectance (where light reflects directly at the camera) that would prevent accurate analysis (Figure 2.4). Luminance change and subsequent camouflage was assessed using the visual system of peafowl (*Pavo cristatus*). Peafowl possess a visual sensitivity (violet-sensitive) similar to that of many shorebird species, who are a common predator of shore crabs (Crothers,

1968). Due to the experimental backgrounds being achromatic, carapace colouration (e.g. hue and saturation) were not analysed as there is no prediction here regarding direction of any colour change, and any change in colour is likely to be irrelevant (Mynott, 2019; Carter *et al*., 2020). ROI mean luminance (perceived object brightness) was calculated using the predicted double cone response, which is considered to underpin the achromatic vision of many bird species (Osorio & Vorobyev, 2005). This was achieved through converting multispectral images from camera colour space to a model of peafowl vision using the Batch Multispectral Analysis Tool developed by Troscianko and Stevens (2015. This mapping approach is regarded to be equally, or more accurate for modelling predicted photoreceptor stimulation in comparison to similar approaches that instead rely on reflectance (Stevens & Cuthill, 2006; Troscianko & Stevens, 2015). Discriminability was then calculated using the absolute difference between the crab and its background using the ROIs to determine the accuracy of background matching achieved, as seen in Stevens *et al.,* 2013 and Carter *et al.,* 2020. A resulting low value would imply there is little discriminability between the two, and therefore suggest higher camouflage efficacy.

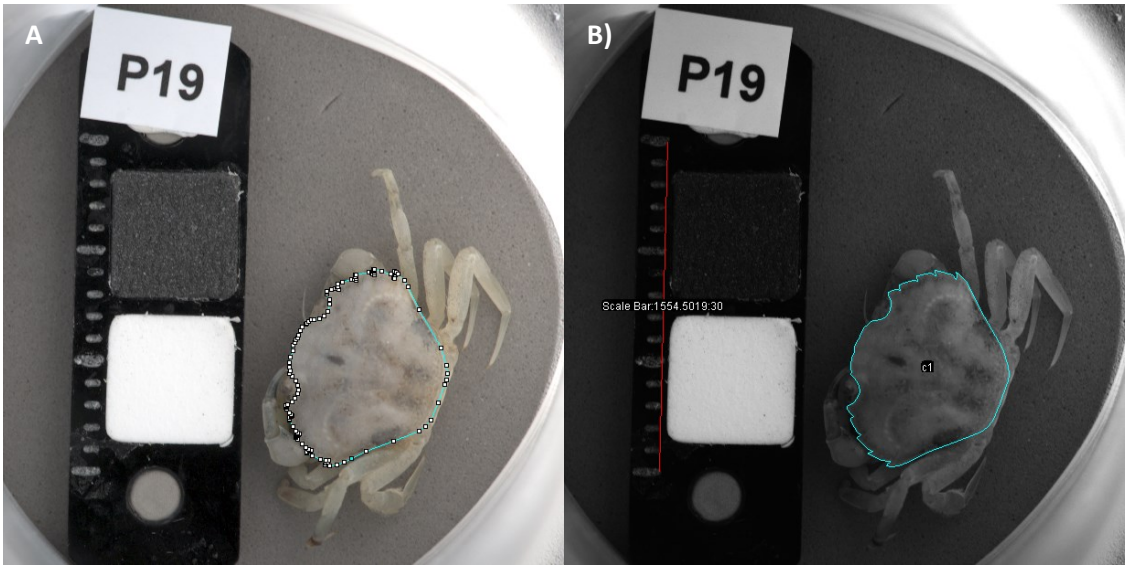


Figure 2.4 Image analysis using imageJ Batch Multispectral Analysis Tool. A) Region of Interest (ROI) selected under the Visual tab reflecting actual colouration of specimen. B) ROI selected under the aligned linear tab for analysis of carapace luminance. A multi-point ROI was used to capture the outline of the carapace as accurately as possible. Standardised shape coverage was omitted in favour of accuracy.

Tissue Processing and Analysis

The euthanisation procedures used at the end of this study reflects those outlined in Chapter 2 under the same title.

Statistical Analysis

Of the original 88 shore crabs collected, 79 were included in the final statistical analyses (14°C: 19 control, 21 plastic; 24°C: 19 control, 20 plastic). The majority of those omitted from statistical analysis were due to individuals becoming >25mm in diameter (carapace) during the acclimatisation period. Carapace sizes exceeding 25mm indicate the individual has reached sexual maturity (adulthood), and therefore will exhibit reduced phenotypic plasticity. Unfortunately, a small number were targets of cannibalism despite efforts to keep individuals separated.

Statistical analyses were carried out using R v.3.6.1 (R Core Team, 2019). The data generated from multispectral images in week two were preferentially used during statistical analysis over week one due to reduced stress levels experienced by individuals General Linear Models (GLMs) were used to test for the effects of temperature and microplastic ingestion on background matching, luminance change, and growth (weight and carapace diameter). Temperature (14°C and 24°C) and treatment group (control and plastic) were combined in the model to produce 4 levels of treatment (C14, P14, C24, P24). The appropriate GLM family and link were determined using visual inspection of quantile-quantile plots, residual distributions, residual vs fitted value plots, and the skewness function (e1071 package v.1.7-3, TU Wien, Austria) to assess normality. Where changing GLM family was not appropriate, data were transformed to reduce skewing. Information regarding changes to family, link, and data transformations are provided within the results section. Maximal model controlling was used in conjunction with model simplification outlined by Crawley (2011) to produce the most parsimonious model per dataset. Variables controlled for included: moulting, carapace diameter (size), and start size. Tank was not included in the analysis due to collinearity with treatment, resulting in singularities within the model output. As tank was not found to be significant in Chapter 2, this should not affect the results. The Akaike Information Criterion (AIC), Variance Inflation Factor (VIF), and overall model deviance were used in combination to successively remove non-

significant terms. Where appropriate, GLM models were also followed by Tukey least-square mean post-hoc analysis to clarify which level of treatment was significant.

A GLM was not considered appropriate to analyse the time taken to moult, instead a Cox proportional-hazard model (CPHM) survival analysis was performed (Fox & Weisberg, 2011). CPHMs assess multiple factors simultaneously (e.g. treatment, size, tank) and how they affect the rate and probability of an event occurring (Cox, 1984). The minimum adequate model was determined by systematically removing non-significant terms. The 'event' term was set to the occurrence of an individual moulting, with 'censored' being assigned to individuals that did not moult within the duration of the experimental period. Hypothesis test statistics for both GLMs and CPHMs are presented within the results section.

Results

The mean luminance of individuals at the start of the experiment was not found to significantly differ between the four treatment groups (Kruskal Wallis, $\chi^2_{(3)}$ =1.55, p=0.56). This shows that there were no differences in starting appearance between groups.

Week Two

Analyses for this time point were conducted on non-moulted crabs exclusively (n=68), to assess the degree of short-term luminance change and subsequent background matching within moults. Tank was removed as a model variable due to collinearity with treatment, resulting in singularities within the model output. Tank was not found to be significant in Chapter 2, and therefore should not affect result interpretation.

Luminance Change

Mean luminance change data exhibited a strong positive skew with the addition of negative values. Data was log transformed with the use of a constant (+1.5) to produce a weaker positive skew without negative values. This data was then used in conjunction with a Gamma GLM family and identity link. The mean luminance change of individuals after two weeks was found to be significantly greater after exposure to increased ambient temperature (GLM, $\chi^2_{(3,64)}$ =0.01, p=0.004). A Tukey post-hoc confirmed multiple stressors did not significantly impact luminance change when compared to the control group at 24°C (p=0.95) (Figure 2.5). Microplastic as a singular

stressor also did not significantly impact luminance change (p=0.97) as in Chapter 2. Size, and an interaction between treatment and size were initially controlled for, but not found to significantly affect luminance change (GLM, $\chi^2_{(1,63)}$ =0.001, p=0.25), or the model's overall AIC or deviance and was therefore removed. An interaction between treatment and size was also used, which was also not found to be significant or affect the deviance of the final model when removed (GLM, $\chi^2_{(3,60)}$ =0.03, p=0.44).

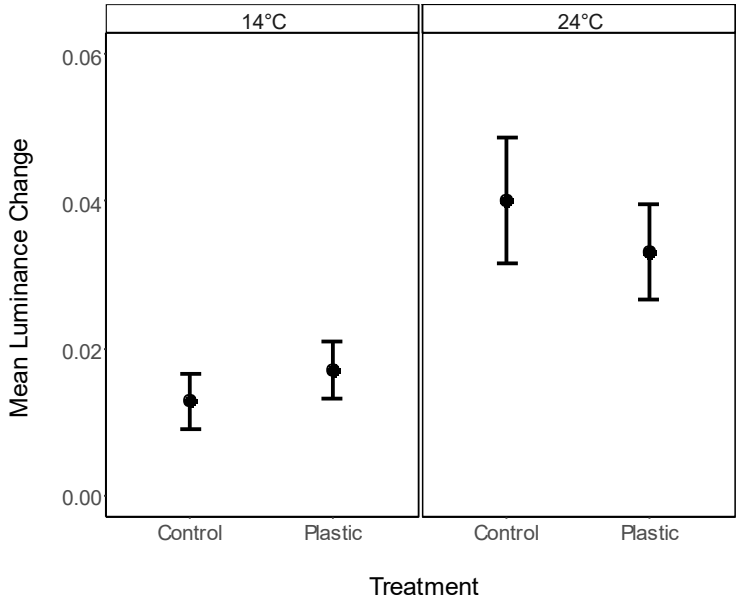


Figure 2.5: Mean luminance change after two weeks. A higher value indicates a greater change. A significant difference was found between temperature groups (p=0.004), with individuals at 24°C changing more than their 14°C counterparts. Only crabs that had not moulted (n=68).

Background Matching

The data were strongly negatively skewed, therefore raw values were reflected and subsequently multiplied to the power of 4. This created a strong positive skew which could then be used in a Gamma family GLM with inverse link. The degree of background matching displayed by individuals after two weeks of exposure was found to be significantly affected by temperature (GLM, $\chi^2_{(3,64)}$ =0.16, p=0.002). Individuals at 24°C were found to match their background more closely (Figure 2.6). A Tukey post-hoc test revealed that multiple stressors however did not significantly affect background matching (p=0.9), and neither did microplastic alone (p=0.98). Size was controlled for in the model initially, however it was found to not significantly affect background matching (GLM, $\chi^2_{(1,63)}$ =<0.001, p=0.95). Size was consequently

removed as it did not affect the model's overall deviance or AIC. An interaction between treatment and size was also included, however this too was removed for the

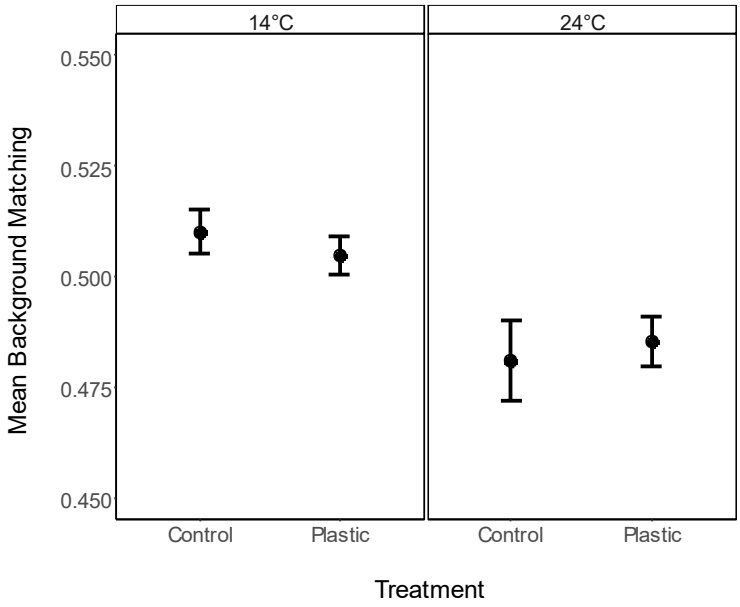


Figure 2.6: Mean background matching after two weeks: A lower value indicates better background matching (reduced difference between subject and background). A significant difference was found between temperature groups (p=0.002), with individuals at 24°C changing more than their 14°C counterparts. Only crabs that had not moulted (n=68).

same reasons (GLM, $\chi^2_{(3,60)}$ =0.02, p=0.55).

Growth

Combined stressors did not appear to have a significant effect on the weight change during the first two weeks of the experiment on non-moult crabs (GLM, $\chi^2_{(3,64)}$ =0.002, p=0.55). A Tukey post-hoc test showed that microplastic ingestion as a singular stressor also did not significantly affect weight change (p=0.92), and neither did an increase in temperature (p=0.19). Start size was initially controlled for in the experiment, however size was not found to significantly affect weight change (GLM, $\chi^2_{(1,63)}$ =0.003, p=0.10) and was subsequently removed from the model as it did not affect the model's overall deviance or AIC. An interaction between treatment and start size was also included, then subsequently removed as it did not significantly affect the model's deviance (GLM, $\chi^2_{(3,60)}$ =0.005, p=0.23).

Carapace diameter is fixed between moults, as a result we would not expect to see a change in carapace diameter when no moult had occurred. Due to few crabs having

moulted within two weeks of the experiment start (n=11), changes in carapace diameter were not analysed at this time. Carapace growth across the entire length of the experiment was consequently analysed under the subheading of 'Growth', in 'Week Six' later in this chapter.

Week Six

Luminance Change

Mean luminance change data for all crabs initially showed a strong positive skew with negative values. Raw data was subsequently log transformed with the addition of a constant (+0.6), and then used in conjunction with a Gaussian family GLM and corresponding identity link. The mean luminance change of individuals after six weeks of exposure to multiple stressors was not found to be significantly affected (GLM, $\chi^2_{(3,75)}$ =0.13, p=0.32) (Figure 2.7). A Tukey post-hoc test revealed that temperature did however have a significant effect on mean luminance change (p=0.002), with individuals at 24°C changing more. Microplastic ingestion as a single stressor however did not (p=0.96). Both size, and moulting were controlled for and significantly affected luminance change (Size: GLM, $\chi^2_{(1,74)}$ =1.5, p=<0.001; Moult: GLM, $\chi^2_{(1,73)}$ =0.38, p=0.001). Those that had moulted exhibited greater change and were also more likely to be larger. An interaction between treatment and size was also included, however,

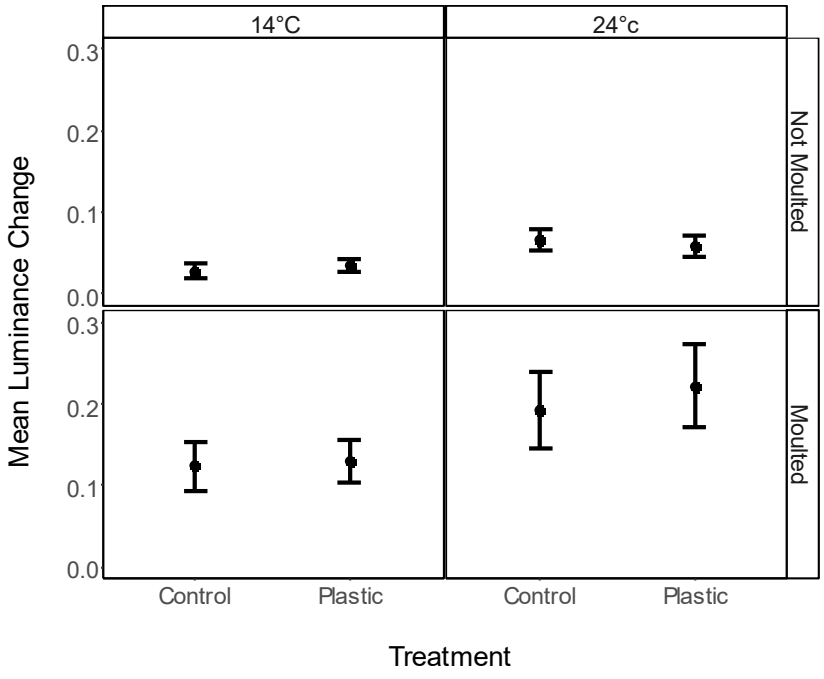


Figure 2.7: Mean luminance change after six weeks: A higher value indicates a greater change. There was a significant difference in luminance change between individuals who had moulted (n=36), and those that had not (n=43) p=0.001.

this was not found to be significant or affect the deviance of the final model and removed (GLM, $\chi^2_{(3,70)}$ =0.05, p=0.68).

Background Matching

Background matching data for all crabs (both moult and non-moult) exhibited a strong negative skew. Data was subsequently reflected, and the resulting values were squared to create a positive skew which could then be used in a Gamma family GLM with identity link. The degree of background matching displayed by individuals after six weeks of exposure was not found to be significantly affected by multiple stressors (GLM, $\chi^2_{(3,75)}$ =0.11, p=0.4). A Tukey post-hoc test did however show increased background matching at 24°C, regardless of moult (p=0.003) (Figure 2.8). Microplastic ingestion alone however did not affect background matching (p=0.99).

Moulting, and size were controlled for in the model, with both found to significantly affect background matching (Moult: GLM, $\chi^2_{(1,74)}$ =2.14, p=<0.001; Size: GLM, $\chi2_{(1,73)}$ =0.14, p=0.05). The effect of size on background matching was however only marginal. An interaction between treatment and size was also included within the model. This was later removed as it was not found to significantly affect background matching and did not affect the model's overall deviance or AIC (GLM, $\chi^2_{(3,70)}$ =0.04, p=0.79).

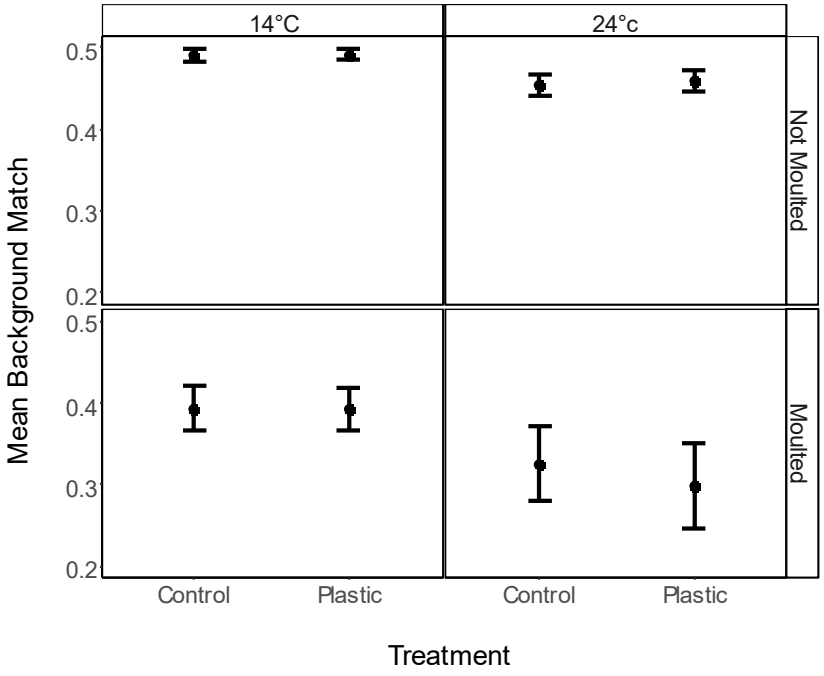


Figure 2.8: Mean background matching after six weeks: A lower value indicates better background matching (reduced difference between subject and background). There was a significant difference in background matching between individuals who had moulted (n=36), and those that had not (n=43) p=0.001.

Growth

Weight change data exhibited a strong positive skew with negative values (weight loss), prohibiting the use of a Gamma GLM. Raw data was therefore log transformed with a constant (+0.8) to meet the normality assumptions of a Gaussian GLM family. Weight change was found to be significantly affected by treatment (GLM, $\chi^2_{(3,75)}$ =0.35, p=<0.01). A Tukey post-hoc confirmed that weight change among the multiple stressors group (24°C plus microplastic) was significantly different from the 24°C control group (p=0.03), and both groups at 14°C (Control: p=0.003; Plastic: p=0.05) (Figures 2.9. C/D and 2.10. A).

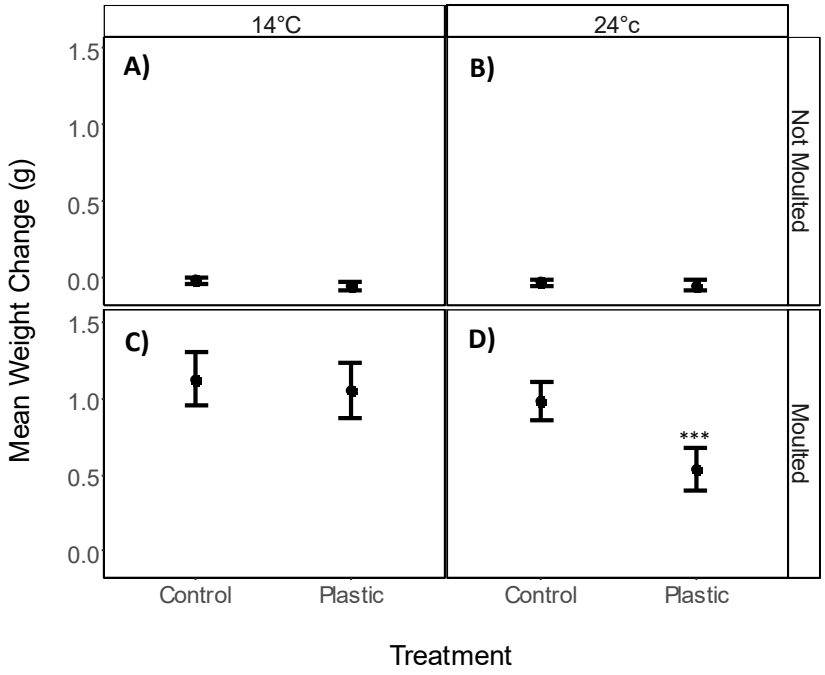


Figure 2.9: Mean weight change (g) after six weeks: A/B) Individuals that had not moulted at 14°C (control: n=8, plastic: n=9) and 24°C (control: n=12, plastic: n=14). C/D) those that had moulted at 14°C (control: n=11, plastic: n=12) and 24°C (control: n=7, plastic: n=6). *** Indicates significantly lower weight gain exhibited by crabs in the plastic treatment group at 24°C than other crabs that had moulted.

Moult, and size were controlled for in the model, with both shown to significantly affect weight change (Moult: GLM, $\chi^2_{(1,74)}$ =2.16, p=<0.001; Size: GLM, $\chi^2_{(1,73)}$ =0.32, p=<0.001). Crabs that had not moulted gained less weight overall, however, there was no significant difference between treatment groups among these individuals (Figure 2.9. A/B). An interaction between treatment and size was also included, which was

found to be significant (GLM, $\chi^2_{(3,70)}$ =0.04, p=0.02). This relates to larger individuals exhibiting greater weight change (typically as a result of moulting). However, this trend is reduced at 24°C with the plastic treatment group gaining less (Figure 2.10. B).

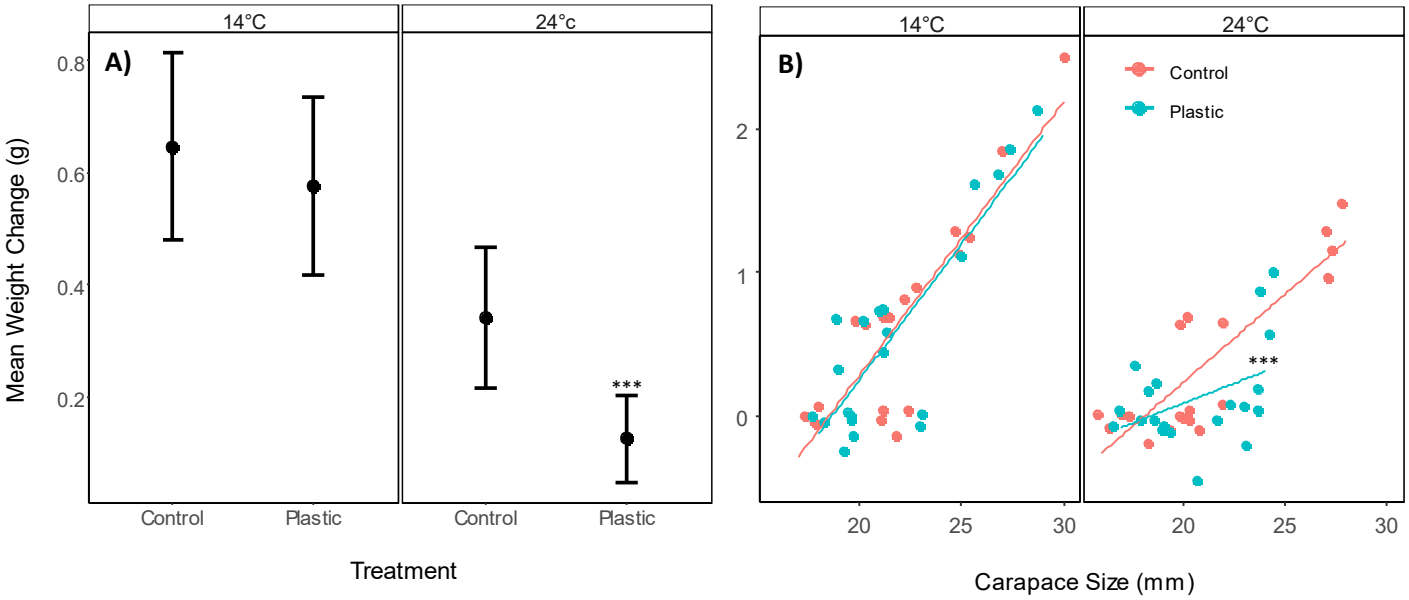


Figure 2.10: Weight change (g) after six weeks: A) Mean weight change (g) of both moulted and non-moulted individuals. B) Weight change by carapace size of both moulted and non-moulted individuals. *** Indicates significantly reduced weight change in the plastic treatment group at 24°C (n=6), when compared to other treatment groups.

Only those that moulted were used for carapace growth analysis, as carapace diameter is fixed between moults. Therefore, we would not expect to see a change in carapace diameter if an individual had not moulted. A Gamma family GLM was performed with corresponding log-link function as raw carapace growth data exhibited a mild positive skew with no negative values. Multiple stressors were found to significantly affect juvenile shore crab carapace growth (GLM, $\chi^2_{(3,32)}$ =0.8, p=<0.001). A Tukey post-hoc confirmed that mean carapace growth was significantly reduced in the multiple stressors group when compared to the 24°C control group (p=0.003), and both groups at 14°C (Control: p=0.001; Plastic: p=0.02) (Figure2.11. A). Start size, and an interaction between treatment and start size were also found to be significant (Start: GLM, $\chi^2_{(1,31)}$ =1.05, p=<0.001; Treatment:Size: GLM, $\chi^2_{(3,27)}$ =0.03, p=0.03), with larger crabs generally growing more per moult, apart from those in the 24°C plastic treatment group (Figure2.11. B). However, these results should be interpreted

with caution due to the small sample size of those that had moulted in the 24°C treatment (control n=7, plastic n=6).

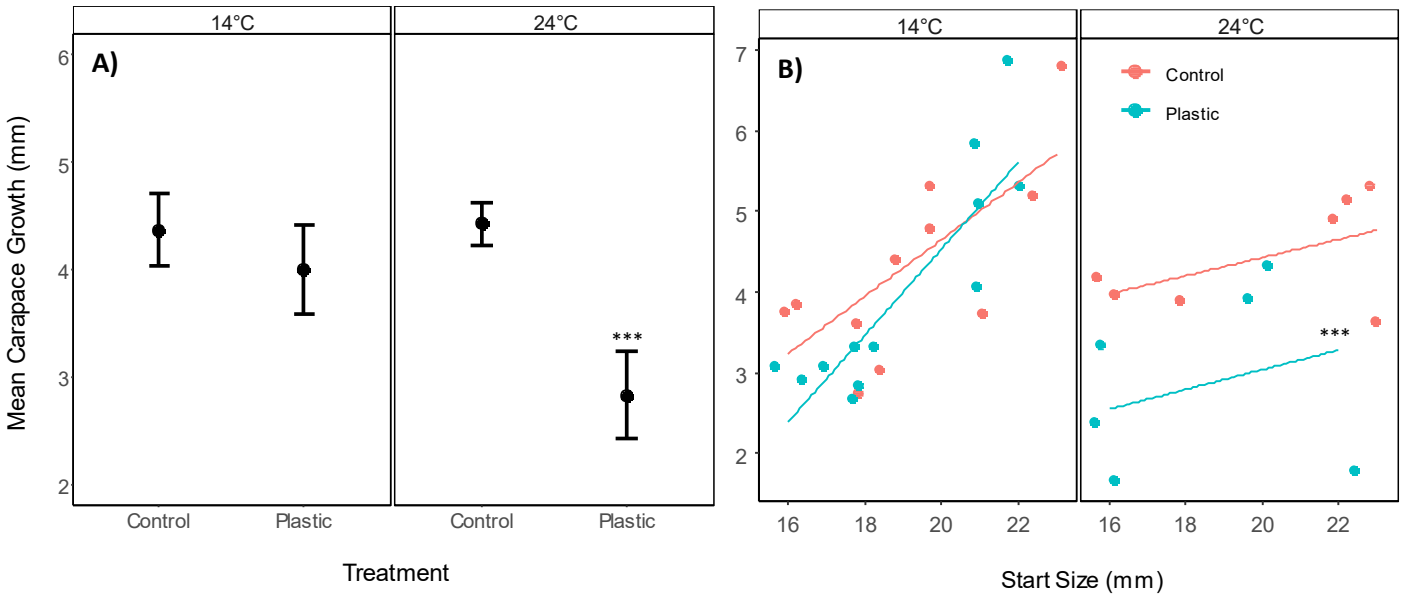


Figure 2.11: Carapace growth (mm) after six weeks: A) Mean carapace growth (mm) in individuals that moulted (total n=36). B) Total growth per individual's starting size (carapace diameter in mm). *** Indicates significantly reduced carapace growth exhibited by crabs in the plastic treatment group at 24°C (n=6), when compared to other treatment groups.

Moult

Combined stressors did not significantly affect the rate or probability of moulting during the course of the eight weeks (Cox Proportional Hazard, $\chi^2_{(1)} = 0.08$, p=0.77) (Figure 2.12). An effect size (hazard ratio) of 0.82 was calculated for microplastic ingestion. An effect size value close to one suggests that there is no difference between the rate of moulting expressed as a result of combined stressors. Temperature did however significantly reduce the probability of moulting (Cox Proportional Hazard, $\chi^2_{(1)}=5.3$, p=0.02). An effect size of 0.6 was calculated for temperature, suggesting that exposure to higher temperatures reduces moult probability.

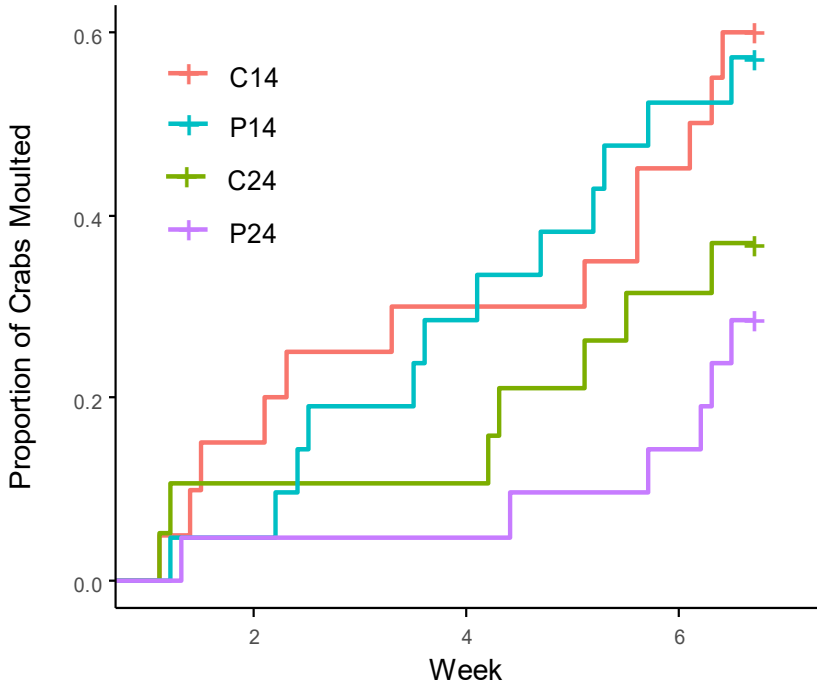


Figure 2.12: Proportion of individuals that moulted: 14°C (control: n=11, plastic: n=12) and 24°C (control: n=7, plastic: n=6). There was a significant difference between temperatures (p=0.02), with fewer moulting at 24°C, and a delay in those that did. A '+' at the end indicates the presence of individuals that had not moulted by the end of the experiment (six weeks).

Microplastic Loading

The overall mean microplastic loading was significantly different between individuals who had moulted and had not moulted, within both temperature treatments (14°C: Kruskal Wallis: $\chi^2_{(1)}$ =76.44, p=<0.001; 24°C: Kruskal Wallis: $\chi^2_{(1)}$ =62.39, p=<0.001). There was also a significant difference between temperature groups, within those that had moulted (Kruskal Wallis: $\chi^2_{(1)}$ =62.39, p=<0.001). Individuals who had not moulted possessed on average 30,967 ±1,425 more particles at 14°C, and 31,154 ±1,431 at 24°C than those that had moulted. The high standard error reflects the variability of miroplastic loading due to individuals moulting at different times, and subsequently shedding their load. Those that moulted in the last week were not included in these calculations as they would not have ingested microplastic spiked feed prior to the end of the experiment.

Discussion

Contrary to our predictions, combined multiple stressors (increased temperature and microplastic ingestion) did not appear to interfere with the luminance change

capabilities of juvenile shore crabs. There was little difference between the control and plastic treatment groups at either temperature, implying that microplastic ingestion and combined multiple stressors did not have a significant effect on luminance change. Mean luminance change was, however, found to be significantly greater at 24°C at both two and six weeks (two-weeks: p=0.004; six-weeks: p=0.002) as seen in Figures 2.5 and 2.7. These results reflect those found by Mynott (2019) who compared juvenile shore crab phenotypic plasticity at similar temperature increments. As expected, the process of moulting did have a significant effect on mean luminance (p=0.001), with those moulting exhibiting greater luminance change (Figure 2.7).

Background matching was also not found to be significantly affected by multiple stressors at either time point (two-weeks: p=0.9; six-weeks: p=0.4) (Figures 2.6 and 2.7). However, background matching did significantly vary in relation to ambient temperature. The absolute difference in mean luminance between the carapace and background was found to be lower amongst individuals at 24°C at both time points, indicating better background matching (Figures 2.6 & 2.8). These results imply that a warmer thermal environment significantly affects the rate at which individuals are able to match their background. As better background matching (and subsequent camouflage) is attained more rapidly at higher temperatures, it is possible that juveniles also evade predation more effectively. While this is essential throughout all life stages, it is particularly vital for settling juveniles who experience increased rates of predation (Moksnes *et al.,* 1998). Furthermore, predators are increasingly active in warmer climates, suggesting that this improved camouflage may be in response to increased predation pressure (Vucic-Pestic *et al.,* 2011). This pressure is further compounded at low tide when shore crabs are more susceptible to visual predators such as shorebirds, which are known predators of crustaceans (Crothers, 1968; Mynott, 2019). Juvenile success therefore relies heavily on the development and employment of effective antipredator defences.

Given that rockpools often exceed the temperatures used in this experiment during summer months due to solar radiation (Thresher *et al.,* 2003) it is likely that a trade-off between camouflage and thermal regulation may take place. Organisms that display phenotypic plasticity typically increase their reflectivity in response to increased solar radiation and environmental warming, thereby mitigating the adverse effects of thermal stress. In doing so, there may be a conflict in displaying an optimal

background matching phenotype, leading the individual to be more conspicuous. This conflict poses significant implications for individual fitness, as poorly camouflaged individuals are more likely to be predated (Stuart-Fox & Moussalli, 2011), and thermal stress negatively impacts individual physiology and metabolism (Paganini *et al.,* 2014).

While this study shows that there were no evident morphological effects associated with exposure to combined multiple stressors at 24°C, it does instead reveal significant physiological effects. By the end of the experiment, individuals in the multiple stressors treatment group had gained significantly less weight than their 24°C control counterparts (p=0.03) (Figure 2.11. A). This is most apparent among individuals that had moulted during the experiment (Figure 2.10. C/D). Furthermore, carapace growth was also found to be significantly reduced in the multiple stressors group when compared to the 24°C control group (p=0.003) (Figure 2.12 A). These results imply the presence of an energetic trade-off between growth and camouflage. Microplastic retention and increased ambient temperature could lead to increased metabolic activity by the individual in an effort to maintain homeostasis (Rankin *et al*., 2019).

Camouflage affects the immediate survival and fitness of the individual (particularly juveniles), as mismatch leads to increased salience, and therefore susceptibility to predation. Growth on the other hand typically holds implications for long term fitness in adults. This is true of fecundity in females, whose reproductive output is positively correlated with body size (Galley *et al.,* 2011). Size is also strongly linked to adult defensibility, with larger individuals more likely to withstand predation events and successfully fend off conspecific competitors. It would stand to reason that camouflage may therefore be favoured in the short-term to increase the immediate fitness of the individual. This is supported by the energy-limited model of tolerance to stress, which assumes that basal maintenance (e.g. energy required for survival) takes priority over other processes such as growth, reproduction, and storage (Sokolova, 2013). This principle has shown to be true of several species (Wieser *et al.* 1988; Kingsbury *et al.,* 2019), with changes in investment reflecting the individual's life stage. The lack of detrimental morphological or physiological effects exhibited in the 14°C plastic treatment group or 24°C control group, is likely due to the singular nature of the stressors. Many organisms are known to exhibit some level of resilience to singular stressors (Lirman & Manzello, 2009; Hughes *et al*., 2017; Rankin *et al*., 2019), however the probability of coping with additional stressors subsequently decreases,

especially if stressors act synergistically (Lange & Marshall, 2017). Our results suggest a need to account for such interactions in future ecological studies and conservation planning.

The responses exhibited to increased temperature in terms of camouflage are indicative of a thermal response curve, where the rate of a process increases with temperature. This trend continues until the given environmental temperature exceeds the individual's tolerance threshold, and subsequently triggers a stress response (Brown *et al*., 2004; Pörtner and Farrell, 2008). While exposure to 24°C did not appear to negatively impact luminance change, the lack of individuals moulting by week six indicates that the warmer thermal environment may have triggered a stress response resulting in the inhibition or delay of moulting. Significantly fewer individuals moulted at 24°C ($p=0.02$) with a calculated effect size of 0.6, further indicating a reduction in the rate of moulting at 24°C (Figure 2.13). This is likely in relation to the aforementioned energetic trade-offs between camouflage and other metabolic functions when experiencing stress. Thermal stress has been shown to affect the production of crustacean hyperglycaemic hormone (CHH) (Chung *et al.,* 1999; Chung & Webster, 2005). CHH has several functions including the regulation of sugar levels in the hemolymph, and the inhibition of ecdysteroid synthesis (moult inducing hormones) (Chung *et al.,* 1999; Chung & Webster, 2005; Chung & Zmora, 2008). Arapid increase in CHH production could consequently be responsible for, or at least contribute to, a delayed timing of moults through altering the production/uptake of other hormones such as ecdysteroids and moult inhibiting hormone (MIH).

As a species, adult shore crabs are able to tolerate a broad range of temperatures, ranging from below freezing to as high as 35°C (Cuculescu *et al.,* 1998; Thresher *et al.,* 2003). This is reflected in the gradual range expansions of shore crabs from their native European waters, as far as California, the southern tip of South Africa, and Australia (Le Roux, 1990; Jensen *et al*., 2002; Ahyong, 2010). It is unclear whether the temperature range at which juveniles operate optimally may be more limited than their adult counterparts (Kelley, 2011). However, juveniles are assumed to have greater sensitivity to environmental stressors (Sokolova *et al.,* 2012). It is also likely that populations which occupy different geographic ranges differ in their thermal optima and threshold tolerance in relation to local environmental conditions.

The shore crabs used in this study originated from the intertidal regions of Cornwall, UK – while many locations will likely experience a greater range in temperatures than those tested here, the experiment is designed to emulate the average conditions experienced by shore crabs at present, and those likely to be experienced in the near future as predicted by climate change models. Additionally, it is unlikely that these juveniles had ever been exposed to such temperatures for periods in excess of six hours. As such, thermal stress is likely to have significantly influenced the results found in this study, but exposure to these conditions may not necessarily invoke the same reaction in individuals that originate from warmer climates. Nevertheless, the interaction of thermal stress and microplastic ingestion/retention as synergistic stressors did indeed negatively impact juvenile growth. However, it remains unclear precisely which stress related mechanism is responsible for the observed disruptions (e.g. energy deficits, or hormone imbalance). Furthermore, it is possible that the significant difference in microplastic loading between temperature groups ($p=0.001$) could be related to thermal stress. Among those that had not moulted, individuals at 24°C had retained significantly more microplastics, particularly on their gills. Work by Kratina *et al.* (2019) suggests that secondary microplastic ingestion negatively impacts respiration in the freshwater amphipod *Gammarus pulex*, and that higher temperatures intensify this effect, reducing respiration rate further. Reduced respiration over extended periods affects ATP production and subsequent energy allocation. It is possible that the increased microplastic burden is a result of individuals being unable to successfully clear sequestered particles from their gills using their flabella (gill rake), or scaphognathite (pumping organ) (McMahon & Wilkens, 1983; Cavey *et al*., 1992) due to increased energy demands.

Future projections estimate there is a 90-99% probability of an increase in the frequency of days that exceed 35°C (maximum air temperature), as well as an increase in the frequency of extended heatwaves (IPCC, 2014). Similarly, projections of microplastic abundance suggest a four fold increase by weight from present estimates (Isobe *et al*., 2019). Coastal regions therefore remain among the most at risk given their increased proximity to microplastic sources, and that shallow marine ecosystems have little buffering capacity against climate change. The predicted increase in magnitude of both stressors demonstrates the need to further research their interactions (amongst others) as they become globally inevitable. Given that

phenotypic plasticity is an antipredator response exhibited by numerous species (Caro *et al.,* 2016), further work should assess their capacity for resistance or resilience in response to a changing world. By determining the ecological effects of combined anthropogenic stressors, and the mechanisms that permit or constrain adaptation, more informed environmental management decisions can be made (Lange & Marshall, 2017).

Chapter 4: General Discussion

Research Findings and Implications

Marine ecosystems are increasingly being exposed to a multitude of anthropogenic stressors, causing rapid environmental changes that can alter community composition (Crain *et al.,* 2008; Doney *et al.,* 2012; Wu *et al.,* 2017). In areas of high anthropogenic activity (e.g. coastal regions) cross-over between stressors (e.g. noise, acidification, microplastics, ocean warming) is not uncommon (Lange & Marshall, 2017). Anthropogenic stressors affect marine ecosystems in different ways, making their interactions problematic as they can amplify the intensity of exposure effects (Byrne, 2011). A significant proportion of existing research pertaining to microplastics as stressors is based on the incidence of microplastic in the environment and within species (Norén, 2007; Lechner 2014; Nelms, 2018; Ferreira *et al.*, 2019) as well as direct physiological consequences (e.g. growth, fecundity, and embryonic development) (Wright *et al.*, 2013; Ziajahromi *et al.,* 2018; Messinetti *et al.*, 2018). However, few examine the effects on invasive invertebrate species, or the antipredator mechanisms that many of these species share, particularly with reference to intermediate juvenile stages. Fewer still, address these in the context of multiple stressors.

This thesis set out to address these knowledge gaps through investigating the effects of microplastic ingestion as a single (Chapter 2), and combined stressor alongside environmental warming (Chapter 3) on juvenile shore crab survival. Specifically focusing on luminance change for camouflage, and juvenile growth (within and post-moulting) as short and long-term survival proxies. Through exploring how these facets of survival are affected by singular and combined stressors, it enables us to understand the degree to which this intermediate life-stage may be affected now and in the long-term, and how these stressors might be mitigated. In doing, so we gain a better understanding of how other marine species that share these environments may be affected. Therefore, allowing us to better predict the level of resilience likely to be exhibited by species that share these traits, and the potential implications of ongoing long-term exposure. From here, broader community responses such as changes in community composition and predator-prey dynamics can also be predicted.

Camouflage and Survival

Colour change is a common antipredator defence among many taxonomic groups (Duarte *et al.,* 2017). Colour change for camouflage typically entails the individual altering some aspect of their external phenotype in relation to hue, brightness, or colour to reflect those of their immediate surroundings (Thayer, 1986; Nokelainen *et al*., 2017; Michalis *et al.,* 2017). In doing so, the individual may be overlooked or misidentified by a potential predator, and subsequently evade predation (Hughes *et al.,* 2019). Colour change occurs through two primary means: morphological and physiological. Physiological changes typically occur over a period of milliseconds to hours, whereas morphological changes are far slower and take place over days to weeks (Umbers *et al.,* 2014). Although it has never been quantified, the process of colour change itself is assumed to be metabolically costly (Talloen *et al*., 2004). Particularly in relation to pigment anabolism and catabolism (morphological), and chromatophore pigment migration (physiological), which require increased energetic expenditure, thereby depleting the individual's overall energy budget (Bergstrom *et al*., 2012; Rogers *et al*., 2013; Duarte *et al*., 2017; Siegenthaler *et al.,* 2018). This increase in energetic expenditure is evidenced by heightened metabolic rates in newt larvae (*Lissotriton boscai*) that assumed darker pigmentation to match their background

(Polo-Cavia & Gomez-Mestre, 2017). As such, this process is susceptible to being affected by physiological stress which can elicit changes in hormone secretion, as well as energy budgets (Thompson & Bayne, 1974; Webster, 1996).

Exposure to anthropogenic stressors such as shipping noise has been shown to induce a stress response of shore crabs, which is generally associated with an increase in metabolic rate (Wale *et al.,* 2013). Given the aforementioned costs of colour change (Bergstrom *et al*., 2012; Rogers *et al*., 2013; Duarte *et al*., 2017), and the limited nature of energy budgets, it is possible that the efficacy of such phenotypic changes would be consequently reduced when an individual is exposed to a stressor. For example, the nutritional status and energy reserves of *Pararge aegeria* larvae dictates the colouration and size of the adult butterfly. Larvae that were exposed to drought-stressed conditions (e.g. heat stressors and limited food availability) were found to be smaller and paler as adult butterflies due to the high costs associated with pigment development while exposed to long-term environmental stressors (Talloen *et al.,* 2004). Physiological stress responses in crab species may also affect hormone secretion, such as those involved in regulating the contraction and dispersal of pigments. This would consequently disrupt the individual's ability to control carapace pigment distribution and their overall appearance with respect to changes in brightness or colour (Webster, 1996; Duarte *et al.,* 2017). This is supported by Carter *et al.,* (2020), who found that prolonged exposure to shipping noise reduces luminance change in shore crabs, as well as carapace growth, and moulting which are all hormone mediated responses.

Across both chapters 2 and 3 it has become increasingly clear that this is not the case for microplastic as a single, or as a combined stressor. Microplastic ingestion was not found to significantly affect luminance change and subsequent background matching. It is possible that the effects of virgin microplastic retention on phenotypic plasticity may require more time to become evident. For example sequestered microplastics reduce the surface area available for gas exchange, thereby reducing gas exchange. Over an extended period this can lead to metabolic depression, a shift from aerobic to an anaerobic metabolism, and the subsequent depletion of the energy reserves (Anestis *et al.,* 2007). Furthermore, the quantities retained in the given time may not have been sufficient to elicit a physiological stress response, either through changes in hormone secretion (Webster, 1996), or alteration to energy budgets (Thompson &

Bayne, 1974). As camouflage affects the immediate survival and fitness of the individual, it is likely that if there were energetic deficits caused, camouflage would be prioritised over other functions. In doing so, individuals would be able to reduce their immediate risk of predation, thus increasing their chances of surviving into adulthood. In the long-term it is unclear whether camouflage would continue to be favoured over other functions (e.g. growth and fecundity), and may be subject to change depending on life-stage, energy-intake, or acclimatisation to stressors.

Luminance change was however found to be significantly affected by exposure to increased temperatures across both control and plastic treatment groups in Chapter 3. At 24°C individuals exhibited greater luminance change, and better background matching than their 14°C counterparts. This suggests that the process of luminance change is strongly temperature dependent, and as such liable to the influence of strong environmental fluctuations (Mynott, 2019). The observed increase can be attributed to warmer environments being more conducive to rapid pigment production through higher metabolic rates which then facilitate increased luminance change, and better camouflage (Duarte *et al.,* 2017). Temperature dependent processes are not uncommon among ectotherms and endotherms, whose biological functions are predominantly regulated by their external environment (Brown *et al.*, 2004). In the instance of species that reside in warmer climates where predation risk is greater, rapid pigment production will be beneficial for species that rely heavily on background matching as their primary antipredator behaviour (Vucic-Pestic *et al.,* 2011). This benefit also applies to species liable to range expansion or movement in response to environmental perturbation induced by climate change.

It remains unclear whether the differences in mean luminance would continue to become more conspicuous with each subsequent moult. As shore crab luminance change is relatively slow process, once mismatched individuals are at greater risk of predation for the duration of their mismatch, even in the absence of stressors. The speed at which phenotypic changes occur across other species range from several seconds, to multiple weeks (Stevens *et al*., 2014). These changes however will presumably still carry similar energetic costs, and therefore may also be constrained by limited energy budgets (Duarte *et al.,* 2017). Previous work by Breteler (1975) shows that up to 73% of energy gained form food intake is spent on new tissue growth, and that energetic trade-offs may occur in the presence of other metabolically costly

behaviours (e.g. colour change) if food consumption is not adequately increased. This is particularly true if a a warmer thermal environment is to become a permanent feature. The process of thermal acclimation is costly as heat-shock response activation requires a significant energetic investment to produce new proteins, and repair or replace those that have already been damaged (Whiteley & Mackenzie, 2016). How microplastic ingestion and its interactions with other environmental stressors will ultimately affect individuals that utilise similar survival strategies is unclear. Different species exhibit various traits that may alter their capacity to cope with environmental perturbation, making the task of predicting how ecosystems will respond to environmental changes increasingly challenging (Voigt *et al.,* 2003).

Size and Survival

Growth scales strongly with future fitness and fecundity in many ectothermic species (Arendt, 2010). These attributes subsequently underpin demographic and community level processes, providing valuable insight into how vulnerable coastal ecosystems may respond to their changing environment (Jaramillo *et al.,* 2017). The measures of size used in Chapters 2 and 3 (weight gain and carapace diameter), are considered to be reliable indicators of an individual's overall fitness, and future success (Reid *et al.,* 1997). In crustaceans growth occurs as a result of consecutive moults which are governed by the cycling of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) through stimulating ecdysteroid synthesis (Chung & Webster, 2003). These hormones are also responsible for regulating hemolymphatic glucose, reproduction, and stress responses within crustaceans (Fanjul-Moles, 2006). Many marine species become hyperglycemic in response to stressor exposure (e.g. pollutants and being outside of their thermal optima), resulting in the increased circulation of CHH and MIH. Abnormal fluctuations in MIH and CHH are recognised as having the capacity to interfere with energy availability, and consequently its investment in essential behaviours, and metabolic functions such as growth (Kim *et al*., 2013). Furthermore, the microplastics used in this study have the capacity to leach toxic endocrine disrupting compounds from the additives, plasticisers, and stabilisers used in plastic production. Low levels of chronic exposure are generally not considered to be lethal. However, they have been shown to significantly reduce feeding, and

subsequently deplete energy stores (Wright *et al*., 2013; Cole *et al*., 2015). As growth did not appear to be affected by microplastic as a singular stressor in Chapter 2, it implies that the quantities sequestered were perhaps insufficient to elicit a stress response. This is likely in part due to the majority of individuals moulting during the experiment and being able to clear microplastics retained on gill surfaces. Additionally, it would suggest that the retained microplastics either did not leach toxic chemical elements, or these chemicals were at sufficiently low concentrations that they did not affect feeding and deplete energy reserves.

Warmer environments are typically conducive to increased growth and elevated moulting rates within crustaceans (Klein-Breteler, 1975; Duarte *et al.,* 2017). However, in the case of Chapter 3, a warmer thermal environment (24°C) did not result in greater weight gain or increased carapace growth in comparison to those kept at 14°C. This was also the case for moulting, in which there was a four-week delay, and an overall reduction in the incidence of moulting at 24°C. In crustaceans, a warmer thermal environment accelerates ovarian maturation, causing ovarian and somatic growth to become antagonistic and resulting in smaller body sizes at maturation (Jaramillo e*t al.,* 2017). The overall reduction in moulting may be due to food availability remaining the same across treatment groups, causing energetic deficits in those kept at 24°C due to increased metabolic activity (Wright *et al.,* 2013). This reduced moulting due to increased metabolic activity is supported by Terwilliger & Dumler's (2001) findings of food availability having the capacity to limit growth to a greater extent than temperature alone. In addition, Watts *et al.,* (2015) similarly found higher food consumption in plastic-fed individuals than control, which is indicative of increased energetic demand.

Weight gain and carapace growth were also both found to be affected by multiple stressors, with some individuals exhibiting net weight loss (Chapter 3). These results support the notion that multiple low-level stressors may act synergistically to elicit a stress response where a singular stressor would not. In this instance, the addition of a second stressor (microplastic ingestion) may have reduced the thermal tolerance range of *C. maenas*, causing an interaction between microplastic toxicity and thermal stress. These results are consistent with previous research, such as the effects of cadmium toxicity in Daphnia being enhanced when present in conjunction with other stressors e.g. increased ambient temperature (Lewis & Horning, 1991). It is also worth noting that lipids constitute around 20% of decapod crustacean's total biomass, and

play a key role in growth and development in larvae and juveniles. When exposed to environmental and anthropogenic stressors, these lipids are used provide ATP in order to fuel the individual's stress response (e.g. a heightened metabolic state), and mitigate cellular damage (Anger, 2001). This provides further weight to the idea that exposure to combined stressors may elicit a stress response which can affect energy allocation as well as availability.

Having established that a reduction in available energy is a likely consequence of exposure to the multiple stressors analysed in this thesis, energetic trade-offs may be required to mitigate potential energy deficits. The results of Chapter 3 suggest that an energetic trade-off may exist between growth and camouflage as luminance change was unaffected by multiple stressors, while growth was significantly reduced. Such changes in energy investment are not uncommon and reflect the priorities of the current life stage of the individual (Wieser *et al.* 1988; Kingsbury *et al.,* 2019). As a juvenile, energetic investment into camouflage would be more favourable as it can increase the immediate fitness of the individual through reducing the risk of predation. This investment however potentially comes at the expense of growth. At earlier instars, this may not incur severe consequences for the individual as camouflage is their primary antipredator strategy (Stevens *et al.,* 2014). Although it is worth noting that this would increase the risk of conspecific cannibalism from larger individuals as shore crabs are not visually guided predators (Robinson *et al.,* 2011). These findings pose the most severe implications for future conspecific interactions, fecundity, and the outcome of predation events.

As adults, larger individuals have greater long-term survival prospects as they adopt a generalist camouflage, and increasingly rely more on physical defences e.g. carapace thickness and aggression (Souza *et al.,* 2011; Stevens *et al.,* 2014). Therefore, smaller individuals are more liable to predation as their probability of successfully escaping a predator is reduces; both in terms of decreased defences (smaller chelae and thinner carapace), and limited energy availability to flee. Similarly, in the case of male-male aggression and competition during the mating season, smaller males are less likely to win altercations, and are more at risk of sustaining life-changing injuries (Souza *et al.,* 2011). In females, a strong correlation exists between reproductive output and body size, with larger females producing greater quantities of eggs and zoae (Galley *et al.,* 2011). The implications of our findings being that in areas

of high stressors cross-over such as coastal environments with high anthropogenic activity, female fecundity may decline affecting juvenile recruitment and future population size, resulting in long-term consequences for population stability.

Mitigating Stressors

Adaptation to environmental change (e.g. climate change) is greatly influenced by a species' tolerance to stressors through plasticity, their potential for dispersal, and the latitudinal ranges they inhabit (Bernhardt & Leslie, 2013). However, the capacity of a species to acclimatise or respond differs vastly among marine invertebrates, which can lead to a range of outcomes, some of which carry severe consequences (Bernhardt & Leslie, 2013). Shore crabs are an example of notoriously successful invasive species, this is largely owed to their flexible responses to stress, and tolerance of large changes in pH and temperature as adults (Tepolt & Somero, 2014). The results from Chapter 2 demonstrate that juvenile shore crabs also exhibit a high level of tolerance to low levels of virgin microplastic ingestion. Both growth and luminance change were found to be unaffected after eight weeks of exposure. This contrasts with existing research from Wale *et al.,* (2015) who found that microplastic ingestion significantly impacts the energy available for growth over four weeks of exposure in adult shore crabs. Additionally, at the opposite end of the life cycle, Woods *et al.,* (2020) also show that microplastic ingestion in American lobster larvae (*Homarus americanus*) leads to a decreased respiration rate, consequently reducing the energy available for metabolic and physiological processes. The differential effects of microplastic ingestion amongst these studies highlight the importance of thoroughly assessing the potential impact across multiple demographics (e.g. life-stages, sexes, populations, closely related species etc.).

While it is possible that the resilience to microplastic as a singular stressor exhibited by juvenile shore crabs is down to their hardy nature. Chapter 2 provides evidence of shore crabs in these intermediate stages being able to reduce their microplastic burden though moulting. By shedding the external epithelial layers of the gill surface, individuals are able to effectively clear retained microplastics, thus mitigating the costs associated with retaining microplastics. Moulting typically occurs more frequently in juveniles, allowing them to reduce their microplastic burdens more effectively than

other life-stages (Crothers, 1968). Further mechanisms also exist in the form of the flabella (gill rake), which sweeps across the gills using setae to dislodge particles (Cavey *et al*., 1992), and the scaphognathite (pumping organ) which reverses the flow of water to displace particles (McMahon & Wilkens, 1983). While these processes are all considered to be effective methods for removing sequestered materials such as heavy metals and parasites from the gill surface (Martin *et al*., 2000), shedding the gill surface appears to be a more effective solution when comparing the microplastic gill burden of those who had and had not moulted in both Chapters 2 and 3. Furthermore, the amount of microplastics ingested orally can also be mitigated as it has been shown that shore crabs have capacity to choose more favourable food items when multiple are available e.g. those with lower microplastic concentrations (Watts *et al.,* 2015). This is significant as microplastics on average take six times longer to reach the excretory phase than food waste (Watts *et al.,* 2014). The delay in excretion provides ample time for toxic compounds to desorb and leach into surrounding tissues.

It is important to consider that there are potential fitness costs associated with immediate plastic responses to mitigate the effects of stressors, which can be further amplified when additional stressors are present (Crain *et al.,* 2008). Many species have evolved under fairly constant environmental conditions or live close to the limits of their environmental tolerances, and may therefore lack the flexibility needed to produce beneficial plastic responses (Somero, 2005). This makes responding to multiple stressors more challenging and decreases the probability of a favourable outcome. In the case of environmental warming, a species' thermal tolerance limit is set by a variety of physiological constraints. These constraints restrict an individual's capacity to adapt and makes them more liable to stress, and the effects of other stressors such as microplastic ingestion (Somero, 2010). Mitigating the potentially detrimental effects of environmental warming comes in a variety of forms, ranging from responses at the cellular level, to responses at a population level. Studies indicate that species-specific differences in metabolism may be the key to coping in adverse environmental conditions, with species that typically possess lower metabolic rates exhibiting greater plasticity (Urbina *et al.,* 2014; Leiva *et al.,* 2015).

The threshold for deleterious effects as a result of environmental warming and microplastic ingestion also varies between developmental stages. Survival is estimated to be significantly lower in juveniles than in adults, and lower still amongst

larvae (Byrne *et al.,* 2010). Species that are unable to respond at a cellular level may be required to move away from environments where stressors are concentrated or overlap e.g. intertidal zones – or utilise seasonal or permanent latitudinal migration to more favourable conditions (e.g. cooler thermal environments). Over time such responses to may become more difficult and less feasible as microplastics are set to become ubiquitous in the marine environment, and the extent to which a species can migrate latitudinally is ultimately limited. Evidence suggests that acclimatisation can occur in response to prolonged temperature increase exposure (Donelson *et al.,* 2012; Tepolt & Somero, 2014). Indicating that perhaps the long-term effects of combined stressor exposure may not be as detrimental as previously thought. However, work by Carter *et al.,* (2020) indicates that this may not be the case for all anthropogenic stressors, with shore crabs demonstrating no obvious signs of acclimatisation to shipping noise after 8 weeks of exposure.

Limitations

As with any laboratory-based study, care should be taken when extrapolating results from experiments conducted within artificial environments as the conditions created may vary to that of any given natural system. Due to the requirements of consistent substrate colouration, temperature, feeding regime, length of experiment, and periodic photography, an in-situ experiment would not have been feasible. Therefore, extensive measures were taken to increase the authenticity and validity of the artificial experimental set-up. This included abiotic conditions such as the light regime, water pH and salinity, ambient temperature, and topography. The ambient temperature of the tanks was recorded daily to ensure significant fluctuations or differences between tanks could be accounted for. Recorded fluctuations in temperature did not exceed ± 0.5°C which would not have significantly affected the thermal tolerance of the individuals at the time.

The microplastics used in both experiments were chosen based on their persistence in the environment and frequent use in industry. However, in some cases they are not among the most commonly found in both, as their usage was constrained by availability for this project. The final master mix consisted of virgin Polyamide (PA), Polyvinylchloride (PVC), Polyethylene Terephthalate (PET), and Polyhydroxy Butyrate

(PHB) fragments. The primary issues with the master mix are that the components were not a range of microplastic types (e.g. fibres, microbeads), and the quantity used. Although the final quantities within feed replicates were reflective of particle abundances recorded in the North Sea (Dubaish & Liebezeit, 2013), it is likely the abundance of particles is greatly underestimated and therefore potentially a conservative quantity for a feeding study (Conkle *et al.,* 2018). As such, our findings may indeed by underpredicting the consequences of microplastic ingestion and multiple stressors in a natural setting. T

he measures taken to reduce microplastic contamination outside of those quantified for study include prohibiting the use of synthetic materials where possible on the researcher's person (e.g. clothing). Additionally, the use of fluorescently labelled microplastics within the samples allowed contaminants to be identified and distinguished from study microplastics during analysis. No evidence of such contaminants was found during the analysis process, this includes no contamination of the control groups following tank swaps to control for tank effects. During the analysis process, microplastics were counted manually using a Leica DM IL LED inverted microscope. Due to the use of manual counting human error needs to be factored into counts, to limit this, five replicates of each sample were counted. The use of an inverted microscope may also affect final microplastic counts in that estimates may be more conservative than actual totals due to particle stratification in wells. Previous studies have found that crustaceans exhibit asymmetry in the microplastics accumulation in gills. This is thought to be related to the scaphognathite pumping mechanism being more dominant on one side of the gill chamber (Watts *et al.,* 2014). Therefore, it is likely that only analysing microplastic counts from the left gill in each specimen may not provide an accurate overview of microplastic burden.

A common criticism of studies that assess microplastic ingestion and retention is that feedstock have been limited to a diet of only microplastics which would influence uptake and nutritional value (Scherer *et al.,* 2017). To combat this issue, feedstock (*M. edulis*) fit for human consumption was sourced from local fishmongers (Seabourne Fish, Cornwall, UK) to ensure minimal contaminants. Mussels were then homogenised and a known quantity of microplastics added with ground crustacean feed (Ocean Free Super Crustanorish sinking pellet) to ensure maximal nutrition.

Future Research

Whilst this thesis addresses several knowledge gaps present within the literature, our findings also give rise to further questions. The window within which juveniles were studied, could be considered quite short relative to their lifespans. Numerous studies have shown that the effects of microplastic ingestion and thermal stress can be highly detrimental during development, and in subsequent life-stages (Wale *et al.,* 2015; Espinosa *et al.,* 2018; Woods *et al.,* 2020). Despite this, there is a significant lack of long-term research monitoring the ongoing effects of exposure from development through to adulthood. Such studies could reveal the true extent of combined stressors on ongoing development, and the associated features of adulthood such as reproduction and conspecific aggression during mating. Moreover, the effects of virgin microplastics may only be evident over extended periods due to their breakdown leaching endocrine disrupting compounds (e.g. bisphenol and phthalates), and toxic heavy metals (e.g. chromium and cadmium) into surrounding tissues (Li *et al*., 2017; Campanale *et al.*, 2020). It is therefore likely that the conclusions drawn from Chapter 2 may be subject to the duration and quantity of particles retained. Repeating this study with microplastics that have been exposed to common environmental pollutants is key to understanding the true risk posed by microplastics, and their interactions with other stressors.

Antipredator behaviour as a broader concept is an area in which the effects of microplastic ingestion and retention have been largely understudied despite their prevalence across multiple taxa. Behavioural responses to predation events such as fleeing, attacking, and visual displays (among others), have as of yet not been analysed. Particularly with respect to potential energy trade-offs and subsequent energetic deficits caused by thermal stress and microplastic ingestion. Given the potential reduction in available energy and depletion of energy stores shown in Chapter 3, we expect that a decline in the effectiveness of antipredator behaviours is a distinct possibility. Such responses have been demonstrated through increased shore crab retreat time in response to a simulated predation event, when exposed to shipping noise (Wale *et al.,* 2013). Berke *et al.,* (2004) also summarise that antipredator behaviours such as camouflage through decorating are in themselves

energetically costly and are sensitive to energetic trade-offs, particularly in the presence of environmental stressors. In order to continue to maximise current fitness, different life-stages and species may have to trade-off various functions e.g. metabolic or physiological. Through assessing which functions are selected over others amongst different demographics and species, this will provide valuable insight into the future stability of populations. In the case of species which are unable to exhibit plastic responses to stressors, migration may be necessary to minimise exposure to negative consequences. Determining the temporal scale over which individuals relocate (e.g. seasonal or enforced migration), could provide a better understanding of species' tolerance limit to stressors at specific locations. This understanding may also provide an indication of how community structure and composition will change over time.

Furthermore, it is unclear how microplastic accumulation compares with the aggregation of other particles such as colloids, and clay minerals in the gills (Watts *et al.,* 2014). Future research should compare the ingestion of microplastic particles of similar sizes and quantities to that of naturally occurring particles. This may indicate as to whether the effects seen within this thesis are as a result of particle retention, or specifically related to the microplastics themselves. This would further reveal whether the addition of temperature as a stressor increases the retention of all particles, or just microplastics. However, studies such as Wen *et al.,* (2018) have shown that warmer environmental temperatures can cause increased microplastic accumulation in other species such as discus fish (*Symphysodon aequifasciatus*). It also provides an opportunity to study to what extent food availability affects the trade-off between growth and camouflage. This is supported by Campanale *et al.,* (2020) who suggest that the nutritional state of the exposed subject affects the degree to which subjects are affected by the toxic compounds which leach out of microplastics.

Reducing the Problem at its Source

Limiting stressor interaction is the primary way in which pressure on marine ecosystems can be alleviated. However, the process of limiting stressor cross-over is not necessarily straightforward when the overarching source of many environmental stressors is climate change. Given that climate change is exacerbated by

anthropogenic activity, it does provide scope for reducing the scale and severity of climate-related impacts through managing key driving factors. Renewable energy and alkalinisation are considered to have the greatest theoretical potential for addressing key drivers of climate change stressors (Gattuso *et al.,* 2018). Renewable energy holds enormous potential through harnessing the energy of waves, ocean currents, and thermal stratification, while systematically phasing-out fossil fuels. In doing so, the energy needs of the world are met, and emission of greenhouse gases will continue to decline steadily to a predetermined baseline. A significant reduction in atmospheric greenhouse gas concentrations will subsequently reduce the need for broadscale alkalinisation. Alkalinisation involves the addition of alkaline substances that neutralize acidity and consume CO_2. While alkalinisation is generally considered to be feasible, and converting to renewables would allow this process to become more site-specific; alkalinisation would require costly long-term management which may act as an monetary barrier for some governments (Paquay & Zeebe, 2013).

If the climate continues to change at the projected rate, it is unlikely that species will be able to successfully adapt to their new environmental conditions as quickly as required. Therefore, future conservation efforts should primarily focus on reducing stressors that interact with those caused by climate change, such as environmental pollution. A specific area that warrants more research is where plastics and climate change intersect, namely the use of fossil fuels. Adopting renewable energy is only half of the picture, as plastic polymer production is reliant on fossil fuel availability. Reducing plastic pollution has greater potential for being managed through moving away from a single-use plastic culture, identifying pollution sources, and removing existing aggregations e.g. gyres. Although no one immediate solution exists, Lau *et al.,* (2020) predict that 78% of the plastic pollution problem can be solved in the near future using existing knowledge.

Identifying the type of stressors interacting in any given environment are important to establishing appropriate management strategies, such as ocean zoning. It also helps inform and manage expectations for various conservation efforts (Crain *et al.,* 2008). Antagonistic stressors particularly create management challenges, most, if not all interacting stressors would require eliminating to achieve substantial ecosystem recovery. In contrast to this, synergisms respond well to removal of a single stressor, frequently resulting in marked environmental recovery despite the remaining stressor.

However, this is dependent on the system having not passed a threshold into an alternative, unstable state (Crain *et al.,* 2008). Synergisms are among the most common stressor interactions, and therefore pose implications for population persistence, and potentially acting as a bottleneck for some species due to increased mortality (Dupont *et al.,* 2010b; Byrne, 2011). By understanding the mechanisms through which stressors arise, this may allow us to interpret and predict where and how stressors interact, whether it be synergistic, additive, or antagonistic. Thereby allowing areas of concern to be identified and focused on for mitigative strategies (Crain *et al.,* 2008).

Concluding Words

This thesis demonstrates that as singular stressors, microplastic ingestion and environmental warming pose a limited threat to the long-term survival of hardy species such as shore crabs. We also demonstrate that oral and ventilatory uptake routes may be important in natural populations which occur in regions of high plastic pollution. However, when these stressors overlap geographically and temporally their interactions present implications that could span across multiple species and life-stages. Shore crabs are an abundant species globally, and play an important role in northern hemisphere food webs (Watts *et al.,* 2015). Through studying their response to common marine stressors, it allows us to deduce how survival mechanisms and antipredator behaviours may be impacted in other marine invertebrates. The economic significance of marine invertebrates should not be understated, not only with regards to their contribution in the commercial fishing industry, but also as a source of new drug candidates (De Zoysa, 2012; Leal *et al*., 2012). Moreover, for their role in food webs, as ecosystem engineers, and general contribution to the marine ecosystem (Leal *et al*., 2012).

The marine environment is in a constant state of perturbation and change due to anthropogenic activity, with common marine stressors increasingly overlapping. This is particularly true of coastal marine ecosystems, which are among the most valuable and heavily used natural systems, but the most at risk from climate change (Jaramillo *et al.,* 2017). They provide essential ecosystem services, including shoreline protection and food from fisheries and aquaculture (Bernhardt & Leslie, 2013). In

September of 2020 the United Nations announced that the international community had failed to achieve any of the 20 Aichi biodiversity targets (Xu *et al.,* 2021Therefore, it is more important than ever to understand how stressors interact, and what their effects are. Through understanding their effects at the individual, community, and ecosystem levels, the scientific community will be better able to gauge the consequences of long-term exposure and whether species may be able to buffer the associated detrimental effects. Though it remains unclear whether individuals will be able to acclimatise to multiple stressors in the long-term, evidence suggests does that acclimatisation can occur in response singular stressors (Donelson et al., 2012; Tepolt & Somero, 2014). However, if nothing is done to reduce the omnipresence of stressors within the marine environment and species fail to adequately adapt, this will have a dramatic impact not only at the community level, but hold the potential for ecosystem collapse. Therefore, it is imperative to limit the presence and cross-over of stressors, as well as ensure new biodiversity targets are met to safeguard the future of the marine environment upon which so many depend.

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